

**Cellular aspects of the immune response of the turbot,  
*Scophthalmus maximus* (L.)**

**by**

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in partial fulfilment for the degree of**

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**Department of Biological Sciences, Faculty of Science**

**In collaboration with  
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## ABSTRACT

Cellular aspects of the immune response of the turbot, *Scophthalmus maximus* (L.).

Amanda Susan Burrows.

Peripheral blood leucocytes of the turbot, *Scophthalmus maximus*, were characterised into 4 distinct groups following morphological, morphometric and histochemical examination. Total and differential cell counts were determined. Thrombocytes, the most abundant leucocyte type (52%), were highly mobile and encountered in several morphological forms. Granulocytes, representing 5.6% of the leucocyte population, histochemically most resembled the mammalian neutrophil. Both large and small lymphocytes (40.8%), were encountered. Monocytes were rarely observed (1.6%). Thrombocytes and monocytes were phagocytic *in vitro* at 12°C and 22°C, showing increased phagocytic activity at the higher temperature. The thymus was paired and consisted of a well developed outer cortex and an inner medullary region. The spleen was bounded by a fibrous tissue capsule and contained a large volume of blood. Diffuse areas of red and white pulp, ellipsoids and melanomacrophage centres were apparent. Lymphocytes, thrombocytes and mature erythrocytes made up the cellular components. The kidney, located beneath the vertebral column contained haemopoietic tissue throughout. Excretory tubules were evident posteriorly. Cellular elements included developing granulocytes, large and small lymphocytes and melanomacrophages. Investigation of ontogenic development of the lymphoid tissue, from 24h post-hatch to the completion of metamorphosis (Day 63) revealed thymic, splenic and kidney rudiments all present at Day 4 with the first lymphoid cells appearing in thymus and kidney by Day 8. Splenic lymphoid cells and the development of areas of white pulp were apparent by Day 28. Differentiation of the thymus had occurred and melanomacrophage centres were seen in the spleen, completing structural lymphoid development by Day 63. Critical stages of lymphoid ontogeny were correlated with easily recognisable external morphological features. A study of the kinetics of carbon clearance by the reticuloendothelial system, revealed a phagocytic capacity in the spleen, kidney and heart. Splenic carbon was seen at 20min post injection, accumulating around ellipsoids and rising to a maximum level at 24h. By Day 5 carbon levels within phagocytes, by now more distant from the ellipsoids, had begun to decrease and carbon was seen within melanomacrophages. Levels of kidney carbon, present within large macrophage-like cells which increased in size forming larger aggregations, increased to a maximum at Day 3. Clearance appeared more rapid in the posterior kidney. Low level uptake was seen within the epicardium. Carbon uptake was not observed in the liver or gill. Kidney leucocyte migration *in vitro* was examined to a range of chemoattractants using a number of assays. 24h bacterial culture supernatants of *Vibrio alginolyticus* induced significant cellular responses. The under agarose assay demonstrated migration inhibition to 100%, 50% and 40% supernatant dilutions. Enhanced migration was detected to dilutions of 5-50% in the microchemotaxis chamber, being optimal at 20%. The leucocyte polarisation assay demonstrated cell orientation in response to 100% culture filtrate and the capillary tube migration assay revealed cellular inhibition at concentrations of 10% & 50%. Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) also induced migration in the filter-based assay, being optimal at 10<sup>-7</sup>M. Cellular migration and orientation were observed in filter and polarisation assays to turbot serum, with normal and activated serum inducing elevated responses in the filter based assay. No response was detected by any of the assay systems to n-formylmethionyl-leucyl-phenylalanine (FMLP) or casein at any concentration tested. Results are discussed in relation to the cellular defence mechanisms of fish.

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## AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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Signed..........

Date.....30 June 1995.....

**To Brad and Emily  
with love**

## CHAPTER 1

### GENERAL INTRODUCTION

#### 1.1 TURBOT

Fish are the oldest group of vertebrates. With over 20,000 species, teleosts represent the largest group, accounting for over half of modern day vertebrate species. Fish represent an important protein source, especially in third world countries. An ever-increasing annual consumption of fish world-wide places enormous pressure on already dwindling wild stocks. Culture of fish is increasingly being utilised to make up the shortfall. Species commercially farmed include representatives of salmonids, carps, catfish, tilapia, sea bass, sea bream and marine flatfish (Manning, 1994).

Turbot belong to the *Scophthalmidae* family of sinistral flatfish and are widely distributed in the Atlantic Ocean and along European coasts (Deniel, 1990). They are considered to be one of the most valuable marine food-fish, prized for their fine flavour (Wheeler, 1978). The biology of this species has been studied by Jones (1972, 1973, 1981), Flowerdew and Grove (1979), Cousin, Baudin-Laurencin and Gabaudan (1987), Deniel (1990) and Skiftesvik (1992) and some aspects of their immunology have also been investigated (Al-Harbi and Austin, 1992; Obach and Laurencin, 1992; Estevez, Leiro, Sanmartin and Ubeira, 1993).

An early study of the feasibility of culturing turbot was made by Purdom, Jones and Lincoln (1972). More recently, Oleson and Minck (1983) and Aguirre, Fernandez-Cortes, Fuentes, Gonzalez-Gurriaran, Labarta, Mora, Perez-Camacho and Perez-Pazo (1989) have studied methods of their cultivation, which is largely centred in warmer Mediterranean regions. According to Estevez and Leiro (1994), turbot is the most economically important species currently farmed in Europe. Spain has seen one of the

greatest increases in turbot production in recent years with over 1300 metric tonnes of fish produced in 1993, a figure which represents 20% of their total fisheries supply (Anon, 1994). Despite the fact that the turbot is close to the limits of its range in northern European waters (Wheeler, 1978), some production does exist in the United Kingdom, in Scotland and the Isle of Man where this high value fish is of increasing economic importance. Significant disease problems in cultured turbot have also been reported (Castric and De Kinkelin, 1984; Schlotfeldt, Ahne, Jorgensen and Glende, 1991; Figueras, Novoa, Santarem, Martinez, Alvarez, Toranzo and Dykova, 1992; Block and Larsen, 1994; Ross, McCarthy, Huntly, Wood, Stuart, Rough, Smail and Bruno, 1994).

## 1.2 DISEASE CONTROL

Disease has often been regarded as one of the principal economic constraints to the expansion and intensification of fish culture (Richards, 1980). Since the effects of disease in the farm situation can be catastrophic, methods of prevention would be highly advantageous. Control measures in intensive farming situations have primarily involved the use of chemotherapy. However, there are significant limitations with the use of chemotherapeutants, including development of resistance, toxicity, adverse environmental effects and ineffectuality against viral pathogens. The advent of effective vaccines for use against fish diseases is therefore an extremely important factor in maintaining the viability of commercial aquaculture. Several commercially available vaccines currently exist for use in fish (Siwicki, Anderson and Studnicka, 1994). In the UK these include vaccines directed against *Aeromonas salmonicida*, the causative agent of furunculosis in salmonids, *Yersinia ruckeri*, the causative agent of enteric redmouth disease (ERM) and *Vibrio* sp., including *Vibrio salmonicida* which causes 'coldwater vibriosis' in farmed Atlantic salmon.



Fish vaccines, reviewed by Ellis (1988a, b) aim to maximise the immunological response should the fish encounter a specific pathogen but to avoid the problem of immunological tolerance. This is achieved by presenting an effective antigen, in suitable form, at the correct developmental stage of the fish (van Muiswinkel, Rombout and Evenberg, 1988). Vaccine administration can be carried out in a variety of ways including immersion of the fish in a vaccine bath, via the oral route and by injection (Ellis, 1989). To date, by far the most effective route of administration has been via the injection route and although time consuming and labour intensive it is routinely used in vaccinating cultured fish against bacterial diseases.

However, for effective vaccines to be developed, a fundamental understanding of fish immune mechanisms is essential (Wardle, 1995).

### **1.3 THE IMMUNE SYSTEM OF FISH**

Literature pertaining to the cells and tissues of the lymphoid system has been included in the respective ensuing chapters of this thesis. This section aims to give an overview of the immune responses of fish which have been extensively reviewed by Cushing (1970), Corbel (1975), Dorson (1984), Lamers (1985), Ellis (1978, 1989), Manning (1994) and Siwicki, Anderson and Studnicka (1994). Fish have been shown to possess an extensive defence system which can be divided into two broad categories, namely non-specific and specific mechanisms. The former can be elicited by a range of different factors including infectious agents and non-antigenic materials. Specific mechanisms involve stimulation of the lymphoid system, resulting in the production of a response directed against a particular molecular structure.

#### **1.3.1 NON-SPECIFIC DEFENCE MECHANISMS**

Non-specific defences of fish have been reviewed by Ingram (1980), Ellis (1981), Fletcher (1982), Alexander and Ingram (1992) and Secombes and

Fletcher (1992). Together, these physical, humoral and cellular factors make up the natural resistance of the fish. The first line of defence includes a number of physical barriers, namely the epithelial surfaces of the skin, gills and gut and their mucus layer coverings.

#### **1.3.1.1 Humoral Factors**

A range of non-specific humoral factors have been recorded in fish. Transferrin, an iron-binding glycoprotein, acts as an inhibitor of microbial growth. Lectins are proteins or glycoproteins which interact with carbohydrates resulting in the precipitation of soluble substances and the agglutination of micro-organisms. Enzyme inhibitors act by neutralising the activity of enzymes produced by micro-organisms. Lytic enzymes, such as lysozyme, act directly upon invading pathogens resulting in cell lysis. Interferon production has been demonstrated in fish following viral infections (De Kinkelin, Dorson and Hattenberger-Baudouy, 1982). C-reactive protein (CRP) is a serum component of many teleosts which, in the presence of  $\text{Ca}^{++}$  ions, reacts with phosphocholine molecules. Such molecules are common components of the surface structures of many pathogens. This reactivity makes CRP an important protective substance (Baldo and Fletcher, 1973).

Finally, complement is probably the most important non-specific humoral factor, due to its multiple role (Sakai, 1992). Fish complement is an enzyme cascade system, comprising approximately 12 proteins, present in the serum and tissue fluids and is biochemically similar to its mammalian counterparts (Nonaka, Yamaguchi, Natsuume-Sakai and Takahashi, 1981a). Its activity is thermolabile (Sakai, 1981; Rijkers, 1982), requires the presence of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  ions and does not react with immunoglobulin from other species (Fletcher, 1982; Rijkers, 1982).

The third complement component (C3) can be activated by either the classical (antibody-dependant) or alternative (antibody-independent) pathways in fish (Nonaka *et al*, 1981a). Indeed, teleost C3 and C5 have been shown to be structurally and functionally analogous with their mammalian counterparts (Nonaka, Natsuume-Sakai and Takahashi, 1981b; Nonaka, Iwaki, Nakai, Nozaki, Kaidoh, Nonaka, Natsuume-Sakai and Takahashi, 1984; Koppenheffer, 1987) inducing a cytolytic attack (Nonaka *et al*, 1981b) as well as having an opsonising role in phagocytosis (Matsuyama, Yano, Yamakawa and Nakao, 1992). The role of complement in chemotactic attraction of fish leucocytes has also been suggested to be important in inflammatory reactions (Griffin, 1984; MacArthur, Thomson and Fletcher, 1985; Obenauf and Hyder Smith, 1985; Suzuki, 1986).

#### **1.3.1.2 Cellular Factors**

Inflammatory reactions occur in fish (Finn, 1970; van Muiswinkel and Jagt, 1984; Roberts, 1989) but are relatively poorly understood (Ellis, 1989). Both acute inflammation (Finn and Neilsen, 1971) and chronic inflammation responses (Timur and Roberts, 1977; Timur, Roberts and McQueen, 1977) have been reported to occur. They involve granulocytes, macrophages and lymphocytes which have been reviewed by Ellis (1977), Rowley, Hunt, Page and Mainwaring (1988) and Hine (1992). Inflammation involves the accumulation, initially of granulocytes and latterly macrophages and lymphocytes. The migration of such leucocytes can be enhanced by host-derived attractants including serum components such as complement (see above), cytokines (Howell, 1987) and eicosanoids (Pettitt, Rowley and Secombes, 1989). Enhancement by pathogen-derived attractants has also been reported (Nash, Fletcher and Thomson, 1986; Wood, 1990; Sharp, Pike and Secombes, 1991).

Macrophages and monocytes, which are thought to belong to the same cell lineage in fish (Ellis, 1989), are highly phagocytic for both inert and antigenic

particulate matter. There is some controversy however, as to the phagocytic capabilities of teleost neutrophils, which morphologically and histochemically resemble those of mammals. Such phagocytic activity by fish neutrophils was reported by MacArthur and Fletcher (1985) and O'Neill (1985), whereas Griffin (1983) suggested that these cells needed to reach a certain developmental stage before they were capable of phagocytosis.

The enhancement of the phagocytic ability of fish macrophages and neutrophils by opsonins, including complement, antibody, lectins and CRP, has been demonstrated (Sakai, 1984; O'Neill, 1985; Matsuyama *et al*, 1992). However, Wrathmell and Parrish (1980) were unable to find any evidence for opsonisation by complement or antibody in the plaice, *Pleuronectes platessa*. Opsonisation is usually associated with the presence of complement and/or Fc receptors which is in itself disputed in fish (Wrathmell and Parrish, 1980; Griffin, 1984; Sakai, 1984; Johnson and Smith, 1984; Secombes, Chung and Jeffries, 1988; Haynes, Fuller and McKinney, 1988). The apparent variability in the occurrence of opsonisation and the presence of complement and Fc receptors between fish species may actually reflect the variability in the source of cells, their developmental stage and their state of activation in the different studies conducted (Manning, 1994).

Fish phagocytes undergo respiratory burst activity similarly to mammalian cells, whereby reactive oxygen species such as hydrogen peroxide, superoxide anion and singlet oxygen and hydroxyl radicals are generated during the phagocytic process (Nash *et al*, 1987; Chung and Secombes, 1988; Secombes *et al*, 1988; Plytycz, Flory, Galvan and Bayne, 1989). The release of these products has been shown to have a toxic affect on fish pathogens (Graham *et al*, 1988; Whyte *et al*, 1989).

Natural or non-specific cytotoxic cells (NCC) have been described in teleosts as possible evolutionary analogues of the natural killer cells (Ellis, 1989).

They are non-phagocytic cells which resemble monocytes morphologically (Evans, Carlson, Graves and Hogan, 1984) and have cytotoxic effects on cell lines (Carlson, Evans and Graves, 1985; Evans, Jaso-Friedmann, Smith, St John, Koren and Harris, 1988) and pathogens including protozoans (Graves *et al*, 1985).

Whether mast cells are present in fish remains uncertain (Ellis, 1989; Manning, 1994). Some speculation has been made that the eosinophilic granulocytes of the rainbow trout, *Oncorhynchus mykiss*, are analogous with mammalian mast cells (Ellis, 1985). This author also reported responses which resembled the Type 1 anaphylactic hypersensitivity of mammals in rainbow trout injected with extracellular products of *Aeromonas salmonicida*.

In mammals, cytokines are known to be involved in the regulation of cell-mediated reactions. There is some evidence for similar involvement of cytokines in fish. Interlukin 1 (IL1) is an initiator of several cascade systems in mammals. Human IL1 can be recognised by peripheral blood lymphocytes of the channel catfish, *Ictalurus punctatus* (Sigel, Hamby and Huggins, 1986). An IL1-like substance has also been isolated from channel catfish monocytes *in vitro* (Clem, Sizemore, Ellsaesser and Miller, 1985) and from carp, *Cyprinus carpio*, epithelial cell lines (Sigel, Hamby and Huggins, 1986).

Interlukin 2 (IL2) is a polypeptide which, in mammals, is produced primarily by helper T-cells and induces proliferation of activated T-cells which bear the appropriate receptors. Proliferation of carp lymphoblasts by supernatants from mitogen-induced leucocytes taken from peripheral blood, pronephros (Caspi and Avtalion, 1984) and mixed leucocyte cultures (Grondel and Harmsen, 1984) suggested that an IL2-like molecule occurs in this species.

Macrophage activating factor (MAF) has been demonstrated in rainbow trout (Graham and Secombes, 1988) which displayed increased macrophage respiratory burst activity when incubated with supernatants from mitogen-stimulated homologous lymphocyte populations. Graham and Secombes (1990) further demonstrated that rainbow trout produce an interferon-gamma (IF $\gamma$ ) like substance. Both MAF and IF $\gamma$  are produced by stimulated T-cells in mammals. A chemotactic factor capable of attracting granulocytes has been detected in carp in response to both mitogenic (Howell, 1987) and antigenic stimulation (Bridges and Manning, 1991). Secombes (1987) detected the production of a putative lymphokine by mitogen-stimulated blood and head kidney leucocytes of rainbow trout which resulted in an increase in macrophage adherence.

### **1.3.2 SPECIFIC DEFENCE MECHANISMS**

Specific humoral and cell-mediated immune responses, characterised by their specificity and anamnestic properties, have been observed in fish and have been well reviewed (see Section 1.3).

#### **1.3.2.1 Humoral Responses**

The structure of fish immunoglobulins (Ig) has been reviewed by several authors including Carton (1973), Corbel (1975), Marchalonis (1977) and Dorson (1981). In teleosts, including the turbot (Estevez *et al*, 1993), the immunoglobulin is a tetrameric form of the IgM molecule which in mammals is a pentamer. Monomeric and dimeric forms of Ig have also been found in teleosts (Clem and McLean, 1975; Litman, 1976; Lobb and Clem, 1981; Elcombe, Chang, Taves and Winkelhake, 1985). It would appear that these molecules are not simply precursors or breakdown products of IgM but have a defined function (Manning, 1994) and in some cases may represent an entirely different sub-class of Ig (Rombout, Bot and Taverne-Thiele, 1989). However, there is no evidence for a change in the class of Ig produced in fish during the course of a response, as occurs in mammals.

The antibody producing cells in fish are now known to be analogues of mammalian B-cells and it is also accepted that fish possess T-cell equivalents. Early investigations by Ellis (1982) into the hapten-carrier effect first suggested that cellular co-operation occurred. Furthermore, Etlinger, Hodgins and Chiller (1976) found that rainbow trout lymphocytes were stimulated by both T- and B-cell mitogens *in vitro*, providing further evidence for their presence in fish. More recently, lymphocyte subpopulations which either lacked or possessed surface Ig (sIg) (analogous to T- and B-cells respectively) have been detected in several species of fish (Secombes, van Groningen and Egberts, 1983; DeLuca, Wilson and Warr, 1983; Sizemore, Miller, Cuchens, Lobb and Clem, 1984; Miller, Bly, van Ginkel, Ellsaesser and Clem, 1987; Thuvander, Fossum and Lorenzen, 1990) using monoclonal antibodies. It has further been shown that these cells are also functional equivalents of their mammalian counterparts and that monocytes act as accessory cells (Sizemore *et al*, 1984; Clem *et al*, 1985; Clem, Miller and Bly, 1991).

Following antigen stimulation, there is a lag phase of approximately one week, depending on temperature, before serum antibody appears in fish. Titres then increase exponentially, reach a plateau and then decay (Rijkers, 1982). The kinetics of this response have been studied by measuring antibody levels as well as the formation of antibody-producing cells (Lamers, 1985). Secondary antibody responses have also been widely reported in fish (Manning, 1994) although Dunier (1985) questioned whether all of these were true anamnestic responses. However, Secombes and Resnik (1984) did report an improvement in the quality of the secondary response in carp. It must be remembered that a range of extrinsic factors also have an effect upon the immune response of fish. Such factors include temperature, nature of the antigen, dose, route of administration, use of adjuvants and antigenic competition (Ellis, 1989) as well as seasonal and environmental effects

(Marc, Quental, Severe, Le Bail and Boeuf, 1995). Diet, in particular vitamin doses, has also recently been demonstrated to influence the immune response (Hardie, Fletcher and Secombes, 1990; Hardie, Fletcher and Secombes, 1991; Thomson, Fletcher, Houlihan and Secombes, 1994). Indeed, Obach and Laurencin (1992) found that the immune response of turbot was impaired by poor diet. The genetic strain (Lund, Gjedrem, Bentsen, Eide, Larsen and Røed, 1995) and developmental stage (Manning, 1994) of the fish is also important and early exposure to thymus-dependant antigens can induce tolerance in fish (Manning, Grace and Secombes, 1982).

#### **1.3.2.2 Cell-Mediated Responses**

As mentioned above (see Section 1.3.2.1), there is strong evidence for the occurrence in fish of cells which are functionally equivalent to mammalian T-cells. Further manifestations of T-cell activity in teleosts include delayed-type hypersensitivity reactions (DTH) and transplantation immunity. There have been relatively few such studies in fish, most having dealt with the latter in the form of skin or scale grafting, to which there is an acute reaction in teleosts (Rijkers, 1982). A DTH response has been demonstrated in rainbow trout to *Mycobacterium tuberculosis* by primed T-cells (Bartos and Sommer, 1981). This reaction has not yet been well characterised and the mechanisms of production and action of cytotoxic and lymphokine factors in fish is still not fully understood (Stevenson and Raymond, 1990).



## 1.4 AIM OF THIS STUDY

The aim of this study was to further our understanding of the cellular defence mechanisms of the turbot with a view to more effective control of diseases in fish. To this end, leucocyte types were identified on a morphological, histochemical and functional basis. The major lymphoid tissues and the leucocyte populations therein were characterised and the location of phagocytic cells of the reticuloendothelial system determined. Ontological development of the lymphoid organs was also investigated and correlated with external morphological features. Finally, *in vitro* leucocyte migration was assessed in response to a range of chemical mediators (chemoattractants) known to induce migration in mammals and fish.

The turbot was the experimental animal of choice due to its high economic value and increasing importance as a cultured species. It was readily available at various stages of development and could be accurately aged.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 FISH

The species used throughout this study was the turbot, *Scophthalmus maximus*.

##### 2.1.1 SUPPLY AND HUSBANDRY

'0' group turbot weighing 175g (mean weight) were obtained from Golden Sea Produce Ltd, Hunterston, West Kilbride, Scotland. Fish were transported in polythene bags containing 12 l sea water saturated with oxygen to marine aquaria at the University of Plymouth or the Institute of Marine Biochemistry, Aberdeen where they were held in isolated tanks for a quarantine period of 2 weeks. During this time water quality was maintained by the use of biological filtration units (Eheim Ltd, West Germany). Fish were then transferred to 500 l polythene tanks, containing recirculating sea water at 13°C ( $\pm 1^\circ\text{C}$ ), where they were kept for up to 2 years by which time they had reached 300g - 500g in weight. Water quality was tested biweekly and maintained at a salinity of 36‰, pH 7.8, ammonia at  $< 0.025\text{mg l}^{-1}$ , nitrite at  $< 0.05\text{mg l}^{-1}$  and nitrate levels at  $< 20\text{mg l}^{-1}$ . Fish were fed daily on either a specially prepared high protein diet (Appendix I) or on trout No 7 pellets (Ewos-Baker, West Lothian, UK) supplemented with fresh fish and mussels (*Mytilus edulis*).

Larval turbot were obtained from Golden Sea Produce (Hunterston) and Ardtoe (Sea Fish Industry Authority, Marine Fish Farm, Ardtoe, West Highlands). These ranged in age from 24h post-hatch up to 108 days and were sampled *in situ* or transported to laboratory aquaria where they were maintained until sampled. The smallest larval fish (age 0-30 days, weighing 0.002 - 0.1g), were too young to survive transportation and sampled on the farm site. Older larvae were fed exclusively on newly hatched *Artemia*

nauplii until they reached a weight of 0.3g (day 49). Fish above 0.1g were maintained in plastic aquaria, with sponge filter aeration, in the laboratory at 16°C ( $\pm 2^\circ\text{C}$ ) with daily changes of up to 50% of the water several hours after feeding. Fish (0.3g - 1.5g) were fed on dried goldfish flake (P Golding Ltd, Winchester, UK) supplemented with newly hatched *Artemia* nauplii (P Golding Ltd), whilst those above 1.5g received No 2 trout pellets (Ewos-Baker) together with juvenile food supplement (Appendix II).

### **2.1.2 HANDLING PROCEDURES**

Fish were removed from their tanks by hand whenever possible to reduce stress. One hand was placed beneath the head and used to hold the undermost pectoral fin close against the body, whilst the other hand was positioned in the tail region enabling the fish to be raised off the bottom in a horizontal position. Holding the fin in this way and maintaining the fish horizontally ensured that individuals did not struggle, eliminating the stress of net capture and use of anaesthetics. Fish were transferred to a wet paper towel on the bench where a damp tissue was placed over their eyes. Turbot would remain under such conditions without obvious signs of stress for several minutes enabling blood samples to be taken before being returned to the water.

A stock solution of the water immiscible anaesthetic amino-p-benzoate (Benzocaine; Sigma, Poole, UK) was made up by the addition to 90% Industrial Methylated Spirit (IMS) at a rate of  $0.01\text{gml}^{-1}$  and diluted to 50ppm with sea water. This was added slowly to fish in sea water until the required level of anaesthesia was attained when ventilatory movements of the operculum had ceased. Fish were killed by overdose in anaesthetic or exsanguination.

## **2.2 HAEMATOLOGICAL STUDIES**

Blood was withdrawn from the renal portal vein of unanaesthetised fish using a 21G needle attached to a disposable sterile syringe. The needle was inserted on the ventral side of the lateral line, close to the caudal peduncle and between neural spines. For terminal bleeding a site much closer to the body cavity was used. By avoiding excessive pressure when drawing blood up through the needle and removal of the needle before expressing blood from the syringe, cell lysis was minimised.

### **2.2.1 PREPARATION OF BLOOD SMEARS**

To prepare smears, 1.8ml of blood were withdrawn into a 2ml syringe containing 0.2ml of 3.2% trisodium citrate (TSC) in distilled water (d.H<sub>2</sub>O). Blood and TSC were mixed immediately but gently by removing the needle, withdrawing the plunger along the barrel to introduce some air and inverting the syringe several times. Providing the fish was caught and handled carefully and rapidly, with a minimum of stress, coagulation of blood samples did not occur.

Initial observations of smears showed large numbers of amorphous, pale staining cells with no apparent nuclei. The occurrence of such cell damage could be avoided by minimising stress of fish during capture and blood sampling procedures and the rapid drying of blood smears.

Smears were prepared by spreading a 10µl drop of uncoagulated blood on an alcohol cleaned microscope slide with the single even "push" stroke of another slide. A slow traverse of the slide resulted in a thin smear. Smears were dried immediately in a warm air stream until completely dry, followed by immediate fixation for 5min in methanol. Smears were also prepared from blood without the use of anticoagulants for comparison purposes. The rapidity of drying in these became even more critical if a high degree of cell lysis was to be avoided.

### **2.2.1.1 Staining of blood smears**

Cells were routinely stained using May-Grunwald and Giemsa stain (MGG) (Appendix III). Attempts to further characterise turbot leucocytes using MacNeal's Tetrachrome stain, (used in mammalian studies to differentiate between leucocyte types), were unsuccessful. Brilliant Cresyl Blue (which stains mammalian platelets), Leishman's, Wright's and Giemsa alone were also tried without success.

### **2.2.1.2 Anticoagulants**

TSC was the anticoagulant of choice, causing least structural damage to the delicate turbot leucocytes when comparisons were made with lithium heparin (15iu, Sarstedt, Leicester, UK) and ethylenediamine-tetra acetic acid (EDTA, 1.5mgml<sup>-1</sup> blood, Sigma). Blood was mixed thoroughly but gently with the anticoagulant since excessive agitation caused undue haemolysis.

## **2.2.2 TISSUE SAMPLING**

### **2.2.2.1 Lymphoid Tissue**

Lymphoid tissues (thymus, spleen and kidney) were removed and fixed immediately in 10ml formol buffered saline (Appendix IV) to minimise the effects of any post-mortem autolysis.

### **2.2.2.2 Tissue Imprints**

The 'cut' face of freshly excised tissue was carefully touched onto a clean, grease-free slide without smudging. This was repeated until imprints were thin enough to dry almost immediately, minimising cell shrinkage.

## **2.2.3 CELL COUNTS AND MEASUREMENTS**

Haematocrit values (packed cell volumes, PCVs) were obtained by introducing freshly collected blood samples into heparinised microcapillary tubes (1.1 - 1.2mm diameter, Gelman-Hawksley Ltd, Sussex, UK) by

capillarity to the three-quarter point. The other end of the tube had been sealed using Critoseal (Sherwood Medical, St Louis, Mo, USA), a commercially prepared plastic. Tubes were spun in a haematocrit centrifuge at 200 *g* for 10min, removed and a Hawksley haematocrit reader used to determine values. These were obtained in triplicate for 20 fish and expressed as the volume of leucocytes per 100ml of blood.

Total leucocyte numbers were determined in triplicate on blood samples from 20 fish. Counts were made after thoroughly mixing a 1:20 dilution of blood in TSC (0.016% in 1.1% saline), using a Neubauer Improved Haemocytometer (Hawksley) and examined under the microscope at x400 magnification.

Differential white cell numbers were based on counting a minimum of 200 leucocytes in MGG stained smears from 20 fish. In mammalian blood smears, polymorphonuclear leucocytes and monocytes tend towards the margins and tail, whilst lymphocytes tend to occur in the central area of the smear. In a badly produced smear it is possible to obtain up to 50% of the leucocytes in the tail region. Since a similar differential leucocyte migration was observed within turbot blood smears the most accurate method of determining counts was to identify and count all leucocytes occurring in 10 fields of view, equally spaced along the length of the blood film (avoiding the extreme lateral edges, the centre and any areas of excessive cell congestion).

Cell dimensions were measured using an eyepiece graticule calibrated with a stage micrometer (Graticules Ltd, Tonbridge, UK) at x400 magnification. Cytoplasm and nucleus diameters were measured and nuclear:cytoplasmic (N:C) ratios calculated for each leucocyte type.

#### **2.2.4 LIVE CELL PREPARATIONS**

Small samples of anticoagulated blood were placed on microscope slides (R A Lamb, London, UK) and mounted with coverslips. Vaseline was used to

seal the edges of coverslips to minimise evaporation. Direct observations of cell structure and behaviour *in vitro* were then made using phase contrast microscopy over a 30min period.

#### **2.2.5 OSMOLARITY OF TURBOT PLASMA AND SERUM**

A range of NaCl solutions (0.1% - 2.0%) were made up to provide standards for a calibration curve using an osmometer (Osmette S Automatic Osmometer, Precision systems Inc, Ma, USA). Freshly collected plasma and serum were placed in the osmometer and the readings taken, compared with the standard solutions. Values obtained for sera and plasma osmolalities were used to prepare solutions isotonic for turbot leucocytes.

Leibovitz (L15, Gibco Ltd) media was made isotonic with turbot serum and leucocytes by the addition of NaCl (1.89ml 17.5% NaCl in 98.11ml L15 media) to give a final osmolarity of approximately 300mOsm (corresponding to an NaCl content of 1.1%). 1.1% saline was used as a diluent throughout.

#### **2.2.6 BLOOD AND TISSUE LEUCOCYTE PREPARATION**

Blood leucocytes were obtained by loading 1ml citrated blood, diluted 1:1 with isotonic L15 culture media onto an equal volume of lymphocyte separation media (Flow) and centrifuged as described below. The external surface of exsanguinated fish was wiped with ethanol and the body cavity opened with sterile instruments within a laminar flow chamber. The kidney, spleen and thymus were located and removed aseptically using a second set of sterile instruments. Organs were carefully disrupted over a sterile stainless steel mesh (100 x 100 $\mu$ m, Locker wire weavers Ltd, Warrington, UK) or in a cooled glass homogeniser using isotonic L15 media containing 300mg $l^{-1}$  L-glutamine. The leucocyte suspension was placed on ice for 5min to allow sedimentation of cell clumps and a single cell suspension removed, counted and adjusted to 4-5x10<sup>7</sup> cells ml<sup>-1</sup> L15. One ml of the cell suspension was

loaded carefully onto 1ml lymphocyte separation media in a sterile centrifuge tube and centrifuged at 650 *g* for 25min.

Leucocytes were collected as a distinct band and were washed twice in isotonic L15 media at 450 *g*, at 4°C for 10min prior to counting and adjustment to the required cell concentration. Viability, determined by trypan blue (0.2% in 1.1% saline) exclusion was usually >95%.

### **2.2.7 SERUM COLLECTION**

Freshly collected blood was placed in a sterile, conical bottomed tube and allowed to clot at room temperature for 1h. The clot was allowed to retract by leaving it at 4°C overnight. Any remaining blood cells were sedimented by centrifugation (600 *g*, 5min) and serum collected using a Pasteur pipette. Freshly collected turbot serum and plasma had a pH of 7.8.

### **2.3 LIGHT MICROSCOPY**

The lymphoid tissues of the turbot were sampled carefully but rapidly to reduce damage to structural features. Samples were placed into 10ml formal buffered saline fixative for 7 days. Samples were taken from water through ascending alcohols to wax in a Hypercentre 2 tissue processor (Shandon) as described in Appendix V. Tissues were orientated and embedded in 5ml molten Fibrowax ultra pure paraffin wax (R A Lamb) at 60°C. Once wax blocks had begun to set they were placed in cold water for several hours to harden. Sections were cut at 4µm using a Jung microtome and floated onto microscope slides using a water bath at 50°C. Sections were dried overnight at 37°C before being brought to water and stained with Haematoxylin and Eosin (H&E) as described in Appendix VI. All sections were dehydrated after staining and mounted with a coverslip using DPX mountant (R A Lamb).



### **2.3.1 ONTOGENIC STUDIES**

For ontogeny studies, turbot larvae were sampled for light microscopy from 24h to Day 63 post-hatch. Fish younger than Day 35 were sampled *in toto*, but beyond this age lymphoid tissue was visualised with a binocular microscope, dissected out and processed separately. In the smallest fish sections were cut as described in Section 2.3 serially through the entire fish at intervals of 10µm.

### **2.4 HISTOCHEMICAL STAINING**

Leucocytes of the peripheral blood and lymphoid tissues were subjected to the following histochemical stains to demonstrate the presence of specific substances or enzymes in individual cells;

**2.4.1 SUDAN BLACK B STAIN** - a diazo dye with a high affinity for lipid molecules, thought to stain the lipid membranes of granules. Several different staining procedures were tried, the best results being obtained with the method of Sheehan and Storey (1947), as described in Appendix VII).

**2.4.2 PERIODIC ACID SCHIFF (PAS) stain** - a general polysaccharide stain to detect carbohydrate associated complexes including polysaccharides, mucopolysaccharides, glycoprotein, mucoprotein, phospholipids and unsaturated lipids (Appendix VIII). Pretreatment of control slides with salivary amylase was used to determine the presence of glycogen.

**2.4.3 ACID PHOSPHATASE** activity was detected using the method of Hayhoe and Flemans (1979) (Appendix IX). This acid hydrolase is present in the lysosomes of many haemopoietic cells.

**2.4.4 ALKALINE PHOSPHATASE** activity was detected using the method of Hayhoe and Flemans (1979) (Appendix X).

**2.4.5 NON SPECIFIC ESTERASE** activity was detected following the method of Yam (1971) (Appendix XI).

## **2.5 ELECTRON MICROSCOPY**

Blood cells and phagocytic activity were examined by electron microscopy.

Blood from 5 fish was collected into heparinised tubes. One ml was spun in a low speed microcentrifuge (3min) to minimise cell damage. Plasma was removed, taking great care not to dislodge the pellet and pelleted cells fixed then treated as other tissues described below:

Blocks of tissue (1mm<sup>3</sup>) were fixed using 3% glutaraldehyde (EM grade, Agar Aids, Essex, UK) in 2% sodium cacodylate buffer (pH 7.2). Fixative was removed and samples post-fixed for 2.5h in 2ml 2% osmium tetroxide (Agar Aids) in 0.1 M cacodylate buffer, pH 7.5 at 4°C. Tissues were then dehydrated through a series of alcohols and embedded in Spurr resin (TAAB, Reading, UK). An outline of this procedure is given in Appendix XII. Ultra-thin sections of resin embedded material were cut on a Sorvall Porter-Blum MT2-B ultramicrotome using glass knives cut on an LKB 7800N glass knife maker. Spreading of sections was enhanced by treating with chloroform. Those sections showing gold or silver interference patterns (60-100nm thickness) were positioned on copper grids (200 mesh size, Polaron Ltd, London, UK) and stained with lead citrate (Reynolds, 1963) and saturated uranyl acetate. Sections were visualised and photographed using a Phillips 300 transmission electron microscope.

## **2.6 PHAGOCYTOSIS**

### **2.6.1 *IN VITRO* PHAGOCYTOSIS**

#### **2.6.1.1 Light microscopy**

Ten  $\mu\text{l}$  of blood collected in TSC was added to an alcohol cleaned slide and mixed with an equal volume of Pelikan ink (carbon particle diameter 0.02 - 0.05  $\mu\text{m}$  C11/1431a; Gunter Wagner, Hanover, Germany) diluted 1:20 using plaice Ringer (Appendix XIII). A coverslip surrounded by vaseline minimised evaporation during 2 hours incubation at either 12°C or 22°C. Removal of the coverslip and rapid drying served to halt phagocytic activity. Fixed cells were stained with MGG and viewed to determine whether phagocytosis had occurred.

#### **2.6.1.2 Electron microscopy**

For EM observations, anticoagulated blood (TSC) was spun down (100  $g$ , 30min) to produce a buffy layer of leucocytes. Plasma was removed and leucocytes resuspended in plaice Ringer. 0.5ml of the leucocyte suspension was placed in an Ependorf tube with an equal volume of a 1:20 dilution of Pelikan ink (described above). Following incubation for 2h at either 12°C or 22°C (being turned to resuspend cell suspensions each 30min), cells were pelleted, the supernatant carefully removed and fixative added without disturbing the pellet. Cells were fixed overnight at 4°C, then treated as described in Section 2.5.

### **2.6.2 *IN VIVO* PHAGOCYTOSIS**

Turbot were injected intra-peritoneally (*ip*) with 0.4ml of a 1:5 dilution of Pelikan ink (C11/143/a, 1:5 dilution in 1.1% saline = 20mgml<sup>-1</sup>) in 1.1% saline.

Eleven fish (mean weight = 165g) were injected *ip* with this carbon solution at a rate of 0.048mgg<sup>-1</sup> fish = 8mg carbon per fish. They were sacrificed at 20min, 1h, 4h, 12h, 24h, 48 h, Day 3, Day 5, Day 7, Day10, Day 14. Spleen,

kidney (anterior, mid and posterior), heart, liver and gill were dissected out, fixed, sectioned and stained as described in Section 2.3.

To differentiate between carbon and naturally occurring melanin, sections were bleached for 48h in a 20% hydrogen peroxide solution to discolour the melanin pigment.

## **2.7 PREPARATION OF CHEMOATTRACTANTS**

### **2.7.1 BACTERIAL CULTURE FILTRATES**

*Vibrio alginolyticus* culture supernatant was produced by growing bacteria in meat yeast peptone broth (Oxoid, containing; 0.3% meat extract, 0.3% yeast and 1.0% bacterial peptone in 75% filtered sea water) at pH 6.5, with continuous shaking, at 20°C for 24h. Bacteria were removed by centrifugation at 1000 *g* for 30min, the culture supernatant collected and filtered using a 0.45µm filter. Samples were aliquoted and stored at -80°C. Controls were composed of meat yeast peptone broth alone, incubated and stored under identical conditions. Bacteria and control supernatants were diluted with 1.1% saline to provide the following range; 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 100%.

### **2.7.2 N-FORMYLMETHIONYL-LEUCYL-PHENYLALANINE (FMLP)**

Due to the relatively low solubility of the synthetic peptide N-formyl methionyl-leucyl-phenylalanine (FMLP, Sigma) it was initially dissolved in dimethyl sulphoxide (DMSO) to a concentration of  $10^{-2}$ M. This was further diluted to  $10^{-4}$ M with 1.1% saline without precipitation of the peptide, aliquoted and stored at -20°C. Controls of DMSO alone, were diluted and stored similarly. Chemotactic activity of FMLP was assayed at the following concentrations;  $10^{-4}$ M,  $10^{-5}$ M,  $10^{-6}$ M,  $10^{-7}$ M,  $10^{-8}$ M,  $10^{-9}$ M,  $10^{-10}$ M,  $10^{-11}$ M,  $10^{-12}$ M.

### 2.7.3 SERUM

Zymosan-activated serum was prepared by incubating zymosan A (Sigma, Poole, UK) with fresh turbot serum ( $5\text{mgml}^{-1}$ ) at  $20^{\circ}\text{C}$  for 30min with shaking. Following incubation, zymosan was removed by centrifugation ( $1000\text{ g}$ , 15min) and the serum supernatant collected. Turbot serum heat-inactivated at  $56^{\circ}\text{C}$  for 20min, prior to incubation with zymosan, served as a control. Other controls which were not mixed with zymosan included; freshly collected turbot serum and heat inactivated serum ( $56^{\circ}\text{C}$ , 20min). Aliquots were frozen at  $-20^{\circ}\text{C}$  until required. Serum was tested at concentrations of 100%, 75%, 50%, 25%, 10%, diluted using 1.1% saline.

### 2.7.4 CASEIN

Casein (Hammarsten, BDH, Poole, UK) was suspended in distilled water to a concentration of  $20\text{mgml}^{-1}$ . The pH was increased to 12 using 4M NaOH and left until the casein was in solution. The pH was slowly reduced to 7.2 by the dropwise addition of a saturated solution of sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$ , BDH). To obtain the correct osmolarity, NaCl was slowly added to produce a casein solution in 1.1% saline. Samples were aliquoted and stored at  $-20^{\circ}\text{C}$  until required. 1.1% saline was added to give solutions of  $5\text{mgml}^{-1}$ ,  $2.5\text{mgml}^{-1}$ ,  $1\text{mgml}^{-1}$ ,  $0.5\text{mgml}^{-1}$ , and  $0.25\text{mgml}^{-1}$ . Distilled water treated similarly and diluted with 1.1% saline served as a control.

### 2.7.5 LEUKOTRIENE $\text{B}_4$ ( $\text{LTB}_4$ )

Leukotriene  $\text{B}_4$  ( $\text{LTB}_4$ , free acid, L4761, Sigma), obtained in a  $\text{N}_2$  filled ampoule was stored at  $-70^{\circ}\text{C}$  until required. Once opened it was immediately diluted using Leibovitz L15 culture medium (Gibco Ltd) to provide a range of  $10^{-4}\text{M}$ ,  $10^{-5}\text{M}$ ,  $10^{-6}\text{M}$ ,  $10^{-7}\text{M}$ ,  $10^{-8}\text{M}$ ,  $10^{-9}\text{M}$ ,  $10^{-10}\text{M}$ ,  $10^{-11}\text{M}$  which were maintained in the dark at  $0^{\circ}\text{C}$  and used within 1h. All glassware and syringe needles were rinsed in ethanol and air dried prior to contact with  $\text{LTB}_4$ . Chemoattraction assays were carried out as soon as the

dilutions were prepared due to the highly unstable nature of LTB<sub>4</sub> which oxidises rapidly in air. Freezing of diluted samples overnight at -20°C resulted in reduced chemoattraction and was therefore not routinely undertaken.

## **2.8 ASSAYS OF CELLULAR RESPONSES**

The following assays were employed to facilitate measurement of *in vitro* cell responses:

### **2.8.1 UNDER AGAROSE MIGRATION ASSAY**

This method was based on that used by Nelson, Quie and Simmons (1975). Eight ml of an agarose-gelatine medium prepared as follows were added to sterile tissue culture grade petri dishes (50mm diameter). Dishes from a range of suppliers (Flow, Falcon, Sterilin, Nunc, Corning, Costar) were tested following the initial observation of differential cell migration between substrates.

Acid cleaned glass microscope slides, pre-dipped in a 0.5% gelatine solution (Appendix XIV) were also tried as an alternative to petri dishes. Culture medium composed of 10ml x10 L15, 2.5ml HEPES buffer (Gibco, Paisley, UK), adjusted to pH 8.1, and 37.5ml distilled water was warmed to 60°C and supplemented with 0.5% gelatine. One g agarose (Type I, Type Ia or Type IV, Sigma) dissolved in 50ml sterile distilled water using a boiling water bath and cooled to 60°C, was added to the media and poured into dishes to produce a 1% agarose gel. The following gel concentrations were assessed; 1.5%, 1.4%, 1.3%, 1.2%, 1.0%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5% for their effects on migration.

Sets of 3 wells, 2mm in diameter and 2mm apart were carefully punched in a circular pattern (Figure 1). Ten µl of attractant (at varying concentrations) or

an appropriate control were added to the outer and inner wells of each triplet respectively. An equal volume of cell suspension ( $5 \times 10^7$  cells  $\text{m}^{-1}$  L15) was placed in the central well. After 3h incubation at 20°C, cells were fixed for 1h in 2 changes of methanol and left to dry at room temperature for at least 1h prior to careful removal of the gel. Fixed cells were stained using Giemsa and migration distances measured with the aid of an eyepiece graticule. Plates prepared in advance were stored inverted at 4°C with any accumulated fluid being carefully removed prior to use, using a piece of tissue as a wick.

Chemotactic Index (CI) was determined by calculating the ratio of the distance travelled from the rim of the central well towards the control well, to the distance travelled by cells migrating towards the well containing the test substance i.e:

$$\text{CI} = \frac{\text{Active chemokinetic/tactic migration}}{\text{spontaneous migration}}$$

Extreme care must be exercised during the processing of migrated cells since cells can easily be lost or move beneath a lifted gel giving a false impression of active migration.

### **2.8.2 MICROCHEMOTAXIS CHAMBER ASSAY**

A 48-well microchemotaxis chamber (Neuro Probe Inc., Maryland, USA) (Figure 2) was used to assess migration of leucocytes (Boyden, 1962). It consisted of a bottom plate with 48 wells (each 26 $\mu$ l), threaded posts to hold the chamber in exact alignment, a filter sheet and silicon gasket placed above the filled wells and a top plate with 48 holes (maximum volume 50 $\mu$ l).

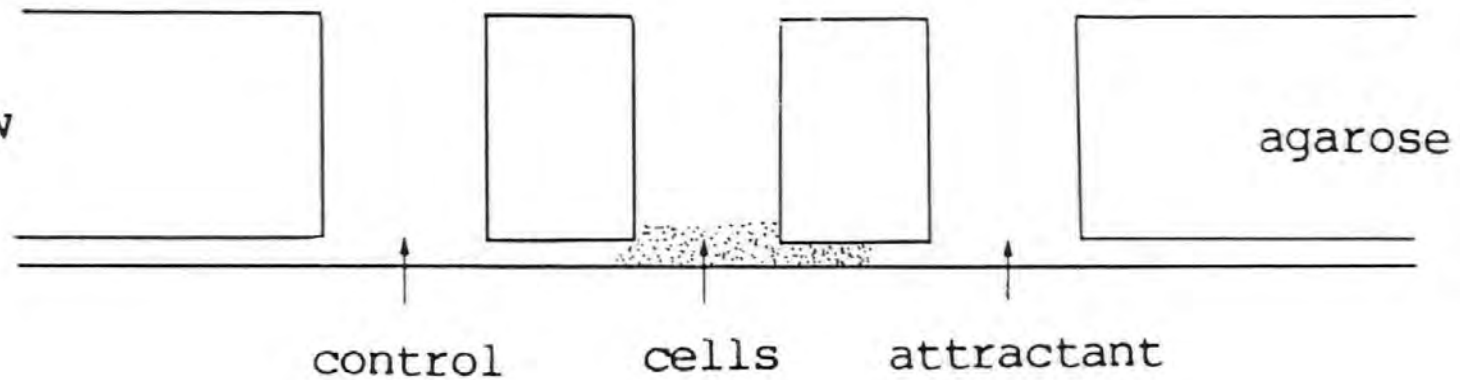
**Figure 1**      **Diagrammatic representation of the under agarose assay.**

**Chemotactic Index of migration (CI) = The ratio of distance A to distance B**

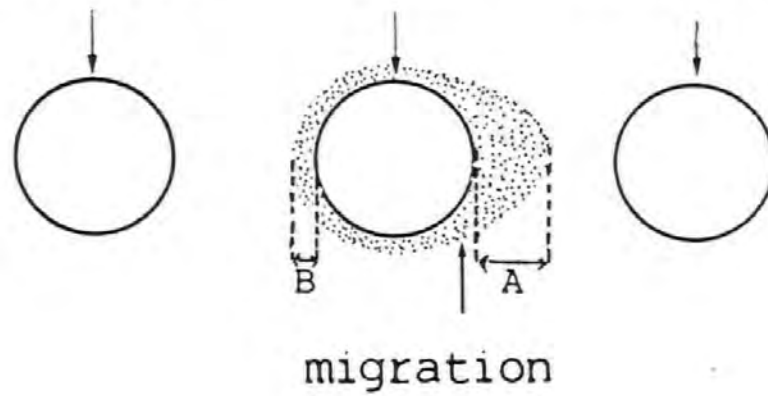
- A      distance of cell migration in direction of attractant**
- B      distance of cell migration in direction of control**



Side view



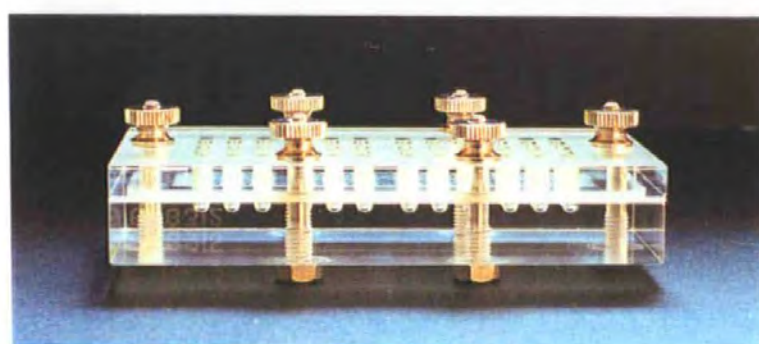
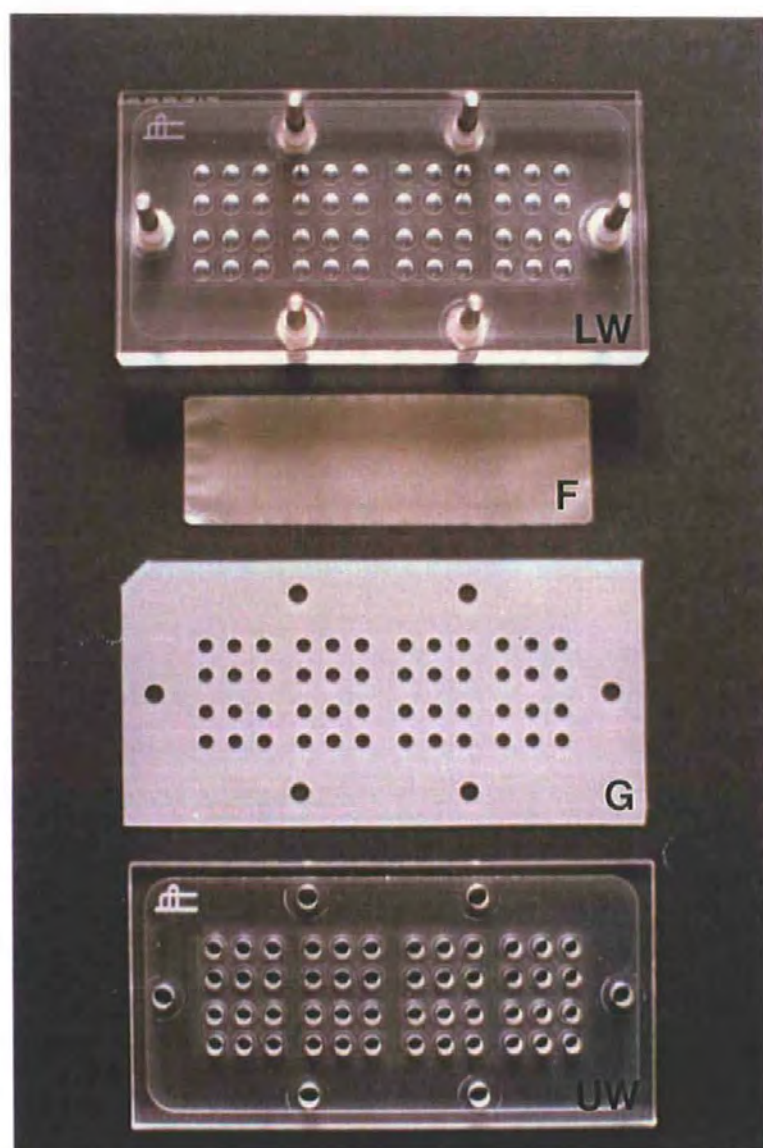
Plan view



**Figure 2      Microchemotaxis chamber.**

**Components:**      Lower wells (LW), PVP filter (F), gasket (G),  
                         upper well section (UW).

**Assembled apparatus is secured by 6 retaining nuts.**



The wells of the lower plate received 26 $\mu$ l of chemotactic / kinetic test solution or a control. These were separated from the upper wells by a polyvinylpyrrolidone (PVP) free polycarbonate filter sheet (3 $\mu$ m pore size). The filter sheet was carefully positioned on top of the bottom plate using two pairs of curved forceps. The silicon gasket and top plate were placed in position over the six threaded posts and held down firmly while the 6 knurled nuts were finger tightened. Raised rims of the top and bottom wells eliminated movement of chemotactic solutions laterally between wells. Once assembled, 45 $\mu$ l of cell suspension (4-5 x 10<sup>7</sup> cells ml<sup>-1</sup> L15) was added to the upper wells as close as possible to the filter (without actually touching it), to avoid depositing air bubbles which would reduce the number of cells applied to the filter.

Following incubation in a humid atmosphere for 120min at 20°C, the apparatus was dismantled and the filter attached to one of the small clamps. Non-migratory cells remaining on the upper surface of the filter were removed by carefully touching the top surface of the filter onto a petri dish containing 10ml 1.1% saline and removing the excess fluid by drawing the upper surface up, across a wiper blade. The filter was air dried prior to fixation in methanol for 60min and staining in Giemsa for 5min. Filters were mounted on a thick microscope slide (75mm x 38mm, R A Lamb) with coverslips (64mm x 35mm, R A Lamb) and DPX mountant. Cells which had migrated to the underside of the filter were counted in 10 high power fields of view (HPF's) using x400 magnification. Assays were performed in triplicate on cells from individual fish for each test substance. Migration was measured as the number of cells moving through pores to the underside of the filter.

Once disassembled all parts of the apparatus were immersed in distilled water, washed well at the end of the experiment with 7X tissue culture

detergent (Flow) and rinsed thoroughly before drying at room temperature overnight.

### **2.8.3 CAPILLARY TUBE MIGRATION ASSAY**

The assay employed was based on a modification of the method of George & Vaughan (1962).

Blood or lymphoid tissue leucocytes were collected and erythrocytes removed using Lymphoprep. The buffy layer of leucocytes was harvested and washed twice (5min, 500 g) in isotonic L15. Following adjustment to  $5 \times 10^6$  cells ml<sup>-1</sup> viability was assessed by trypan blue exclusion. Haematocrit tubes (Gelman-Hawksley Ltd) were filled with the cell suspension, sealed with Critoseal (Sherwood Medical) and centrifuged (5min, 200 g) to produce a packed layer of leucocytes at the plug surface. Capillary tubes were scored just above the leucocyte / media interface with an alcohol wiped glass cutter. Tubes were snapped sharply to provide a clean break that would not interfere with cellular migration from the capillary tube. Using forceps, the blocked end of the tube was embedded in a small amount of silicon grease placed at one edge of the tissue culture dish (16mm, Sterilin, Teddington UK), ensuring that the cut face remained free from any traces of grease. 0.5ml media, containing the solution under test or a control, was added slowly to avoid disturbing the cells. A coverslip was placed over each well, taking care to avoid air bubbles and the chambers incubated overnight at 20°C.

Migration was determined by measuring the distance of cellular movement from the cut edge of the tube using a binocular microscope. More detailed observation of migrating cells was possible using a compound microscope, although this required the use of shallower chambers. These were prepared by cutting strips of EM modelling wax (Pinnacle hard type wax, Detrey, Weybridge, UK) and cutting 3 holes in the wax using a 15mm cork borer.

Each strip was carefully melted onto a glass microscope slide by holding the base of the slide over a flame until the wax just began to melt, producing chambers 1-2mm deep. After incubation migrating cells could be viewed on all objectives once the coverslip and media within the well had been carefully removed using a syringe needle.

#### **2.8.4 LEUCOCYTE POLARISATION ASSAY**

Measurement of *in vitro* polarisation of turbot leucocytes was based on the method of Haston & Shields (1985).

Isotonic L15 culture medium + 10mM morpholinopropanesulphonic acid (MOPS, Sigma) provided the medium for this assay. Turbot leucocytes were obtained from various lymphoid organs as described in Section 2.2.6. Pelleted cells were washed once in L15+MOPS, counted and the number of polarised leucocytes determined. Cells were adjusted to  $1 \times 10^6 \text{ml}^{-1}$  in 1ml L15/MOPS in conical plastic centrifuge tubes and the various chemotactic factors added as 1ml fractions.

Cells were incubated at room temperature for 30min, after which time they were fixed for 10min in 2ml 2.5% gluteraldehyde in isotonic L15 media. The cells were washed twice (400 g, 5min) in fresh media and finally resuspended in 200 $\mu$ l. Those cells which exhibited a shape deviating from spherical were scored as polarised when observed at x400 magnification. 300 cells were counted from each experimental tube and compared with cells exposed to an appropriate control.

## **CHAPTER 3**

### **CELLULAR COMPONENTS OF THE LYMPHOID SYSTEM**

#### **3.1 INTRODUCTION**

The need to identify the leucocytes of a species before an understanding of their immunological role can be advanced has been underlined by Parish, Wrathmell, Hart and Harris (1986). There have been many studies on the immune mechanisms of various fish species (see Chapter 1). However, often relatively little information exists regarding their leucocytes which are a fundamentally important part of the immune system. Prior to studying aspects of cell-mediated immunity in the turbot a detailed analysis of peripheral blood leucocytes was considered essential.

It is generally accepted that the blood leucocytes of fish show distinct morphological similarities to those of birds and mammals and comprise thrombocytes, lymphocytes, granulocytes and monocytes (Ellis, 1977). Most notable differences in fish are the thrombocytes (being nucleated and much larger than their mammalian platelet counterparts) and granulocytes which vary in their proportion of the total leucocyte population, appearance of their granules and nomenclature used in their description.

Since the nineteenth century Romanowsky stains have provided the basis for present day recognition and categorisation of human blood cells (Hayhoe and Flemans, 1979). Such stains are derived from an eosin and methylene blue mixture dissolved in alcohol, diluted and buffered before use, producing a highly polychromatic image. Many reports exist using the staining properties of these dyes as the basis for identification of the leucocytes of a particular fish species. However, the appearance of cells following Romanowsky staining alone is not a satisfactory or infallible guide to their identity and confusion over the nomenclature and variety of cell types exists

even within the same species. In order to clarify the situation, some authors have resorted to the examination of leucocytes at the ultrastructural level which permits observation of intracellular features and organelles, providing clues to the function and identity of the cell type (Cenini, 1984).

Although the leucocytes of many fish species have been studied from a morphological viewpoint (Rowley *et al*, 1988), many authors have referred to the problems encountered in leucocyte nomenclature and more work is required to investigate the function of leucocytes, not simply their morphology, before mammalian terminology can be used in their description. Only recently has some use been made of the range of histochemical stains routinely employed in mammalian leucocyte classification and description. Such tests, used in conjunction with morphological studies, can provide clues regarding cell type by demonstrating specific substances or enzymes within the cellular structure. Enzyme histochemical techniques act as cell markers, giving an insight into function and helping to differentiate between leucocyte types.

The presence of specific enzymes can be demonstrated by their ability to act upon a substrate. The coloured product resulting from this enzyme-substrate reaction is rendered insoluble and can be visualised, marking the location of enzyme activity. The techniques of dehydration, fixation, freezing and high temperature can all reduce enzyme activity. Time, temperature and pH are all important variables and several variations of each test were often tried before the optimum conditions were found. Freshly prepared blood smears provided the basis for cytochemical tests here to minimise inhibition or deterioration of enzyme activity during storage.

Functional properties were also used to supplement morphological, morphometric and cytochemical investigations. Phagocytosis of foreign material is one of the most important mechanisms for protection against



disease (see Section 1.3.1.2). Inert colloidal carbon was utilised as a model to assess leucocyte phagocytosis and ascertain the effects of temperature on its uptake *in vitro*.

The value of haematological studies in human and veterinary medicine is well known although the application of this field to fish diagnostics is presently impaired by the paucity of comprehensive data. To facilitate the use of haematological indices as an effective and sensitive method to monitor physiological and pathological changes for fish as suggested by Mahajan and Dheer (1979) this situation must be improved. The simplicity of blood sampling could result in widespread utilisation of this technique to determine the health state of an individual and would allow its routine use to check the health assessment of farmed stock. For each species a reference baseline of normal cell parameters must be predetermined if we are to understand cellular interaction and the basic principles of fish defence mechanisms.

Fish have been used as sensitive biological monitors for pollution as measurable changes in physiological parameters are often detectable in response to environmental changes. A thorough investigation of morphometric parameters of the turbot should provide a suitable picture from which cellular changes may be detected in response to physical change, environmental pollution and disease.

The following changes from normal haematological levels found by other authors and listed by Williams and Warner, (1976) resulted from; environmental stress - increased eosinophil counts, handling stress - elevated lymphocyte counts, thermal stress - abnormal lymphocyte and eosinophil numbers, oxygen deficiency - lymphopenia, infections - elevated eosinophil numbers with accumulation around lesions, viral disease - leucocyte and thrombocyte degeneration.

Turbot were handled with great care during blood sampling, since stress, physical manipulation of the fish and anaesthesia can all influence haematological parameters, causing variations in qualitative and quantitative data. With stress minimised during blood sampling, the unnecessary use of anaesthetics was avoided.

Techniques suitable for the classification and enumeration of the cellular components of fish blood have been adapted from several mammalian haematological methods. For an accurate reflection of the *in vivo* situation it is essential to minimise changes in sampled blood. Repetitive sampling was possible from the renal portal vein and withdrawing blood directly into a syringe reduced the time blood was exposed to air, avoiding rapid clotting when anticoagulants were not being used.

The use of anticoagulants prevents clotting and although various substances have been tried in fish, opinion varies regarding which is most effective, with certain anticoagulants altering the staining properties of cells (Ellis, 1976). For this reason several routinely used anticoagulants including ethylenediamine-tetra acetic acid (EDTA), trisodium citrate and heparin were compared for their effectiveness and ability to cause minimal disruption to cell structure and staining.

Smears made for differential counts were prepared without the use of anticoagulants in order to minimise cell lysis. Hesser (1960) found higher levels of cell debris and disintegrating cells in fish blood films when compared to those of mammals, suggesting a fragility of fish leucocytes. White cells may be lost even when only partial clotting has occurred (Mainwaring and Rowley, 1985).

Total white cell counts were undertaken to enable comparison with other fish and higher vertebrate species. Differential cell counts are extremely useful in

mammalian haematology where differences from the normal frequency distribution enable the diagnosis of a specific disease state. Such counts can be achieved from a well made and stained blood smear. A minimum of 200 leucocytes were counted and due to the differential leucocyte migration within a smear (Hayhoe and Flemans, 1979), the extreme edges of the preparation were avoided.

Haematocrit or packed cell volume (PCV) provides a means of assaying the percentage volume of whole blood occupied by erythrocytes and plasma following centrifugation of a blood sample. The use of micro-capillary tubes enables such information to be gained from relatively small samples (2 drops of blood) with minimal centrifugation providing a rapid and reproducible test which could be undertaken in the fish farm environment.

The observation of live cell preparations using phase contrast microscopy was also employed since morphological changes and cellular damage can result from smear preparation. This was particularly useful for studying the behaviour and morphology of moving thrombocytes.

The present study was undertaken with the aim of describing morphological, ultrastructural, cytochemical and morphometric properties of turbot peripheral blood leucocytes. Observation of structure and ultrastructure, measurement of cell diameter, ratio of nucleus to cell diameter, shape and staining properties are used to assist in differentiation between cell types. Histochemical stains and functional tests were employed to provide a clearer understanding of the roles of these cells in the turbot.

## **CHAPTER 3**

### **3.2 MATERIALS AND METHODS**

Sections of the Materials and Methods of specific relevance to this chapter include:

#### **2.1 FISH**

##### **2.1.2 HANDLING PROCEDURES**

#### **2.2 HAEMATOLOGICAL STUDIES**

##### **2.2.1 PREPARATION OF BLOOD SMEARS**

###### **2.2.1.1 Staining of blood smears**

###### **2.2.1.2 Anticoagulants**

##### **2.2.3 CELL COUNTS AND MEASUREMENTS**

##### **2.2.4 LIVE CELL PREPARATIONS**

##### **2.2.5 LEUCOCYTE COLLECTION**

###### **2.2.5.1 Blood and Tissue Leucocytes**

#### **2.3 LIGHT MICROSCOPY**

#### **2.4 HISTOCHEMICAL STAINING**

##### **2.4.1 SUDAN BLACK B STAIN**

##### **2.4.2 PERIODIC ACID SCHIFF (PAS) STAIN**

##### **2.4.3 ACID PHOSPHATASE STAIN**

##### **2.4.4 ALKALINE PHOSPHATASE STAIN**

##### **2.4.5 NON SPECIFIC ESTERASE STAIN**

#### **2.5 ELECTRON MICROSCOPY**

#### **2.6 PHAGOCYTOSIS**

##### **2.6.1 *IN VITRO* PHAGOCYTOSIS**

## **CHAPTER 3**

### **3.3 RESULTS**

#### **3.3.1 HAEMATOLOGICAL STUDIES**

The following characteristics were used to differentiate between various blood cell types; cell sizes, nuclear : cytoplasmic ratios (N:C), shape, structure, staining intensity, histochemical staining and ultrastructure. Using such criteria, 4 major types of turbot leucocyte were identified, namely thrombocytes, lymphocytes, granulocytes and monocytes. Summaries of haematological results, linear cell measurements, N:C ratios, total and differential leucocyte counts are given in Table 1. Histochemical staining properties are summarised in Table 2.

#### **3.3.2 THROMBOCYTES**

##### **3.3.2.1 Light Microscopy**

The most abundant leucocyte type observed in blood smears and live cell preparations were thrombocytes. The relatively large nucleus was surrounded by a distinct rim of pale blue cytoplasm in MGG stained preparations (Figure 3a) and existed in several forms; round, oval or indented appearing almost bilobed on occasion. The thrombocyte showed some similarities to the lymphocyte, although the nucleus of the former stained more intensely. Although rounded forms were encountered, cytoplasm was frequently drawn out, tapering at one or both ends of the cell. It was this form which appeared most mobile during observations of live preparations (Figure 3b and 3c), being able to radiate cytoplasmic pseudopodia. Live cells were capable of a high degree of cell shape alteration which may account for the variability in thrombocyte forms encountered in blood smears. When clotting was observed in live cell preparations using phase contrast microscopy, long threads were seen to spread out from the cell pole. An increasingly tangled fibrous network, assumed to be fibrin was slowly created in which other cells became trapped.

Table 1

# HAEMATOLOGICAL PARAMETERS OF PERIPHERAL BLOOD LEUCOCYTES OF THE TURBOT

	Differential Leucocyte Count * (% Total)	Cell Diameter ( $\mu\text{m}$ ) **	N:C Ratio
Thrombocyte	52.0 ( $\pm 8.7$ )	11.3 ( $\pm 1.0$ )*** x 5 ( $\pm 0.6$ ) n=50	0.67 ( $\pm 0.02$ )
Small	40.2 ( $\pm 8.8$ )	6.0 ( $\pm 1.0$ ) n=50	0.83 ( $\pm 0.02$ )
Lymphocyte			
Large	0.6 ( $\pm 0.5$ )	9.0 ( $\pm 0.8$ ) n=10	0.78 ( $\pm 0.02$ )
Granulocyte	5.6 ( $\pm 4.4$ )	11.0 ( $\pm 0.9$ ) n=30	0.49 ( $\pm 0.05$ )
Monocyte	1.6 ( $\pm 0.9$ )	11.5 ( $\pm 1.4$ ) n=20	0.70 ( $\pm 0.03$ )
Total leucocyte number	1.3 x 10 <sup>5</sup> ( $\pm 1 \times 10^3$ ) mm <sup>-3</sup> blood (n=20)		
Haematocrit (Packed cell volume, PCV)	22.7 % ( $\pm 3.0$ ) (n=20)		

\* Figures represent mean average ( $\pm$ SD) of triplicate counts from 20 fish based on a minimum of 200 leucocytes per sample

\*\* Figures represent mean average ( $\pm$ SD) of triplicate counts from 20 fish based on 10 - 50 leucocytes per sample (given by n)

\*\*\* Measured on elongate axis

Table 2

SUMMARY OF HISTOCHEMICAL STAINING PROPERTIES OF  
TURBOT LEUCOCYTES

	<b>Sudan black B</b>	<b>Periodic Acid Schiff</b>	<b>Non specific esterase</b>	<b>Acid phosphatase</b>	<b>Alkaline phosphatase</b>
<b>Thrombocyte</b>	-	**	-	*	-
<b>Lymphocyte</b>	-	-	-	*	-
<b>Granulocyte</b>	***	***	-	**	*
<b>Monocyte</b>	-	-	**	**	*

\* indicates degree of staining, \* minimum to \*\*\* maximum.  
- indicates no reaction.

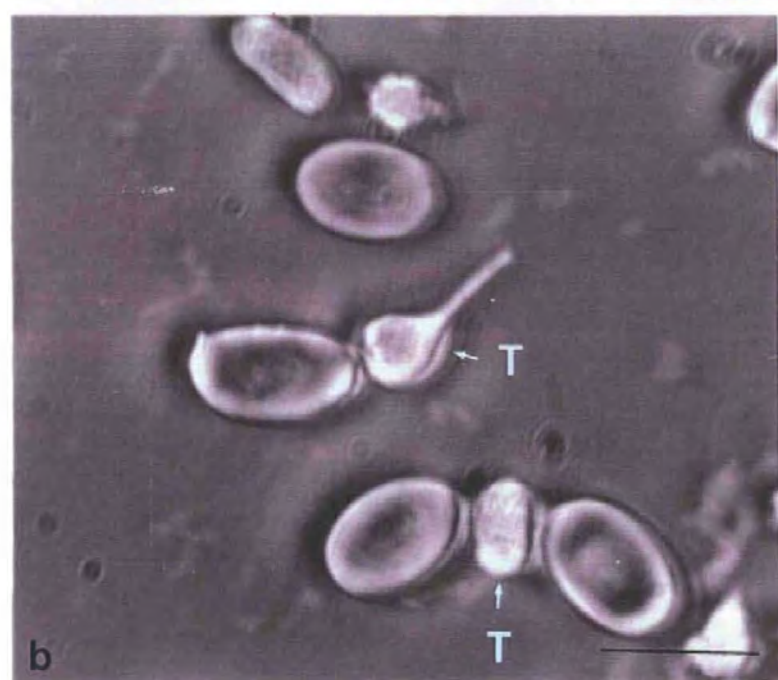
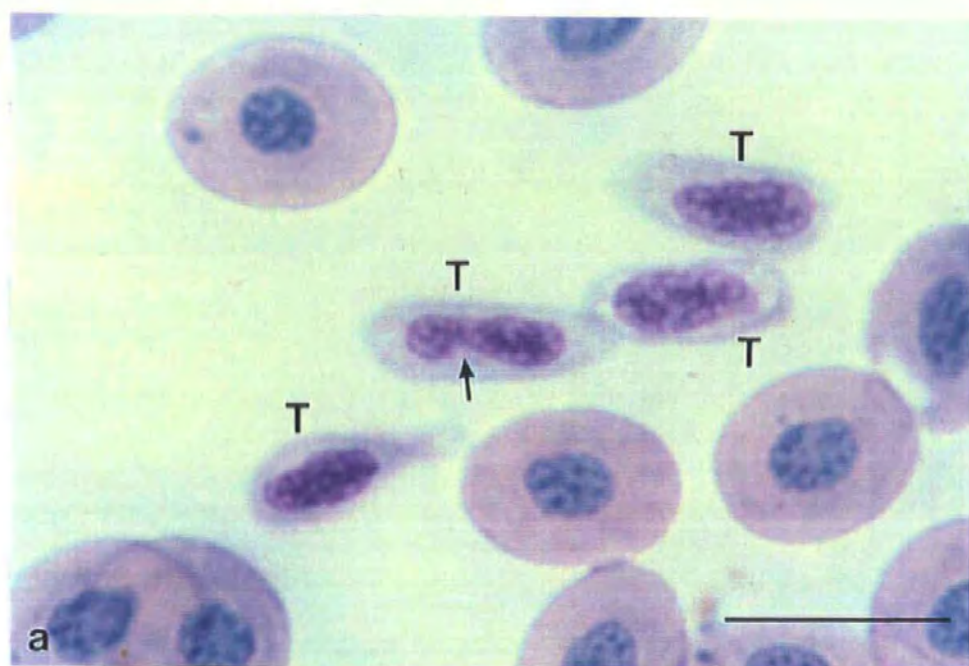
**Figure 3      Thrombocytes**

**a              MGG staining of thrombocytes (T).**

**b and c       Phase contrast micrographs showing *in vitro* movement of  
thrombocytes (T) over a 10min period.**

**(Bar represents 10µm)**





### **3.3.2.2 Histochemistry**

Numerous fine and coarse granules of PAS positive material were dispersed throughout the cytoplasm of thrombocytes (Figure 4a). All morphological forms showed positivity with PAS. Larger, scattered granules of acid phosphatase activity localised within the cytoplasm were also noted (Figure 4b).

All other histochemical stains were negative.

### **3.3.2.3 Electron Microscopy**

Thrombocytes were the most abundant cell type within leucocyte pellets. They exhibited an elongate, fusiform shape when cut longitudinally and were circular in transverse section. The nucleus was sharply indented, centrally positioned with very dense chromatin at its periphery (Figure 5a).

The most prominent cytoplasmic features were large electron lucent vesicles (Figure 5b) caused by numerous, deep infoldings of the plasmalemma. Connections between these vesicles and the cell surface were seen in some sections. Cytoplasmic vacuolation was used to distinguish between transversely sectioned thrombocytes and the lymphocytes they resembled.

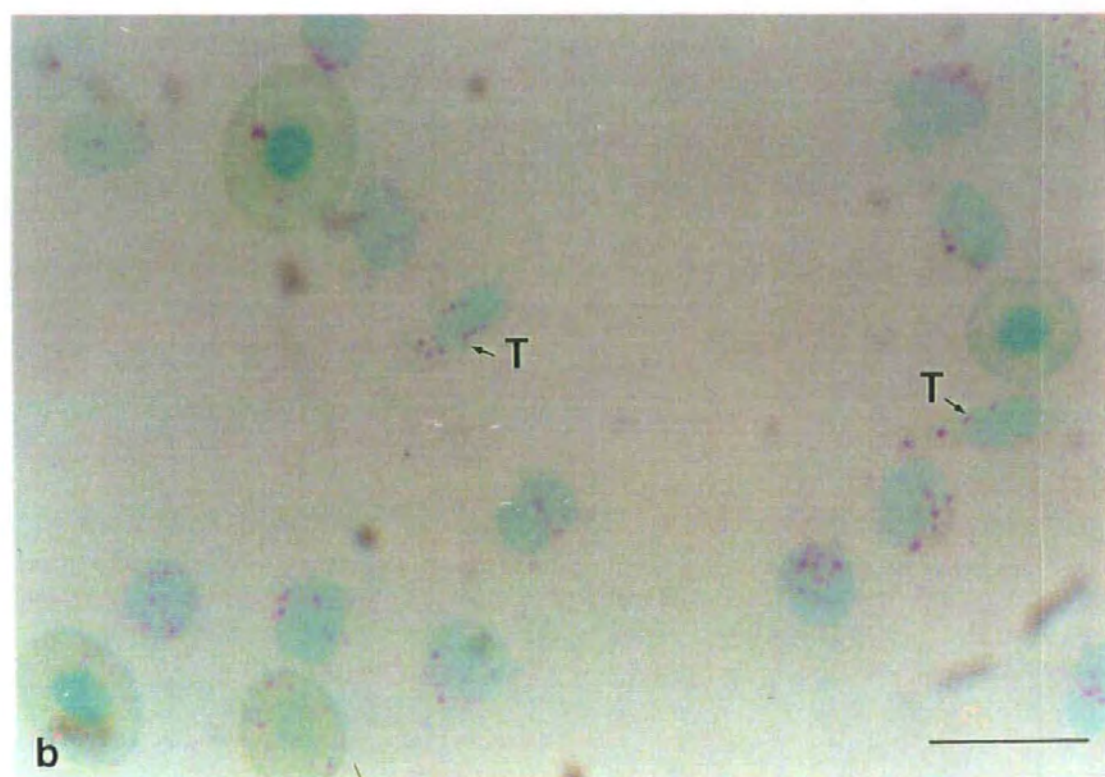
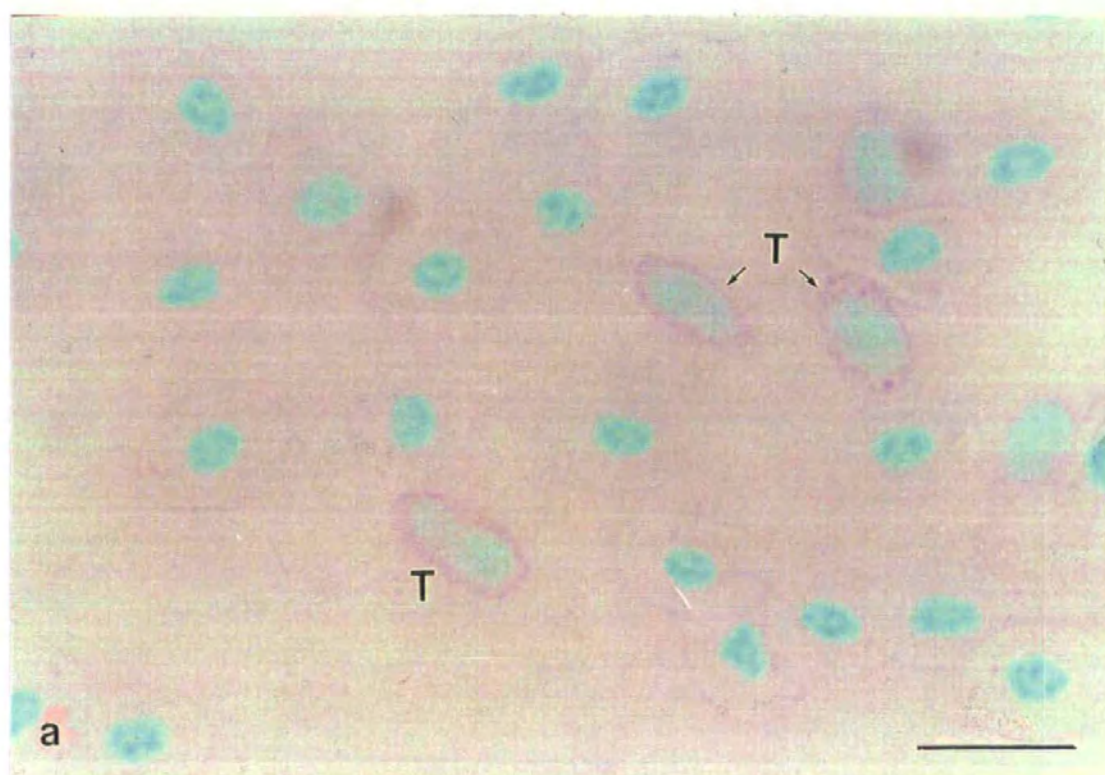
A single electron dense granule was frequently observed within the cytoplasm (Figure 5b). A few clusters of free ribosomes and several mitochondria were normally present and some elements of rough endoplasmic reticulum (rER) were seen. Occasionally, well developed and prominent Golgi complexes surrounded by numerous small vesicles occurred close to the cell nucleus.

Bundles of microtubules were seen below the plasmalemma, often concentrated at the cell poles, in thrombocytes sectioned longitudinally. In transverse section they appeared to encircle the cell (Figure 5b).

**Figure 4      Histochemical staining of thrombocytes.**

- a            PAS staining showing positively stained thrombocytes (T).**
- b            Acid phosphatase staining showing positively stained thrombocytes (T).**

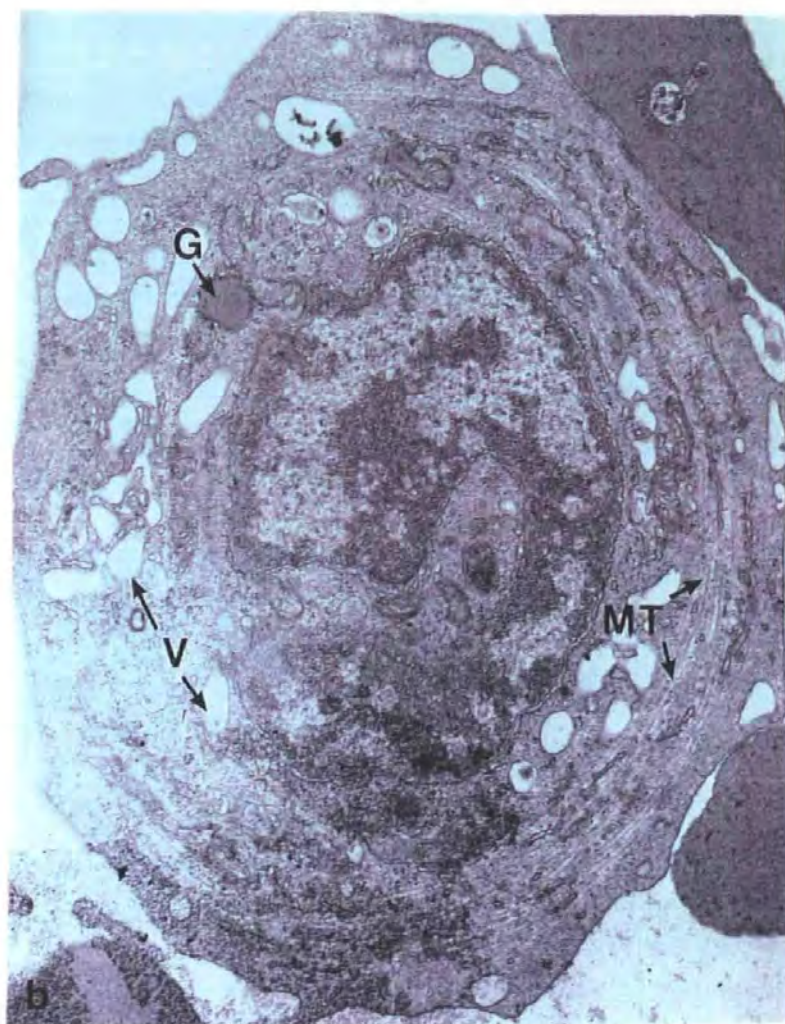
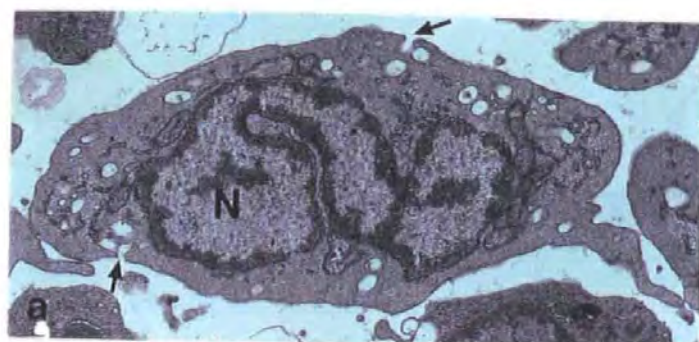
**(Bars represent 10µm)**



**Figure 5      Electron micrographs of thrombocytes**

- a            Longitudinal section through a thrombocyte showing sharply indented nucleus (N).      Arrow indicates infolding of plasmalemma. (Mag x 7,080)**
  
- b            Transverse section illustrating cytoplasmic vacuoles (V), a single electron dense granule (G) and microtubules (MT). (Mag x 25,100)**
  
- c            Well developed Golgi complex (Gc) close to the nucleus. (Mag x 9,740)**





Two types of small granule were seen, one electron lucent assumed to be glycogen and the other, more electron dense assumed to be lysosomal.

#### **3.3.2.4 Phagocytosis**

A large number of carbon particles were observed within the cytoplasm of thrombocytes exposed to carbon *in vitro* (Figure 6a). Carbon was also observed attached to the outer surface of the thrombocytes.

Exposure to carbon resulted in many thrombocytes containing carbon particles within much enlarged cytoplasmic vacuoles (Figure 6b) at both 12°C and 22°C. Pseudopodial - like projections were evident extending to encircle aggregations of carbon (Figure 6c). Carbon uptake occurred to a greater extent at the higher temperature. Vesicles appeared to merge resulting in the presence of fewer very large carbon laden vesicles (Figure 6c, Inset). Such was the amount of carbon taken up by some cells, that the nucleus appeared displaced towards the periphery of the cell. At the EM level engulfed cellular remains from disrupted cells were also seen within cytoplasmic vacuoles of this cell type.

### **3.3.3 LYMPHOCYTES**

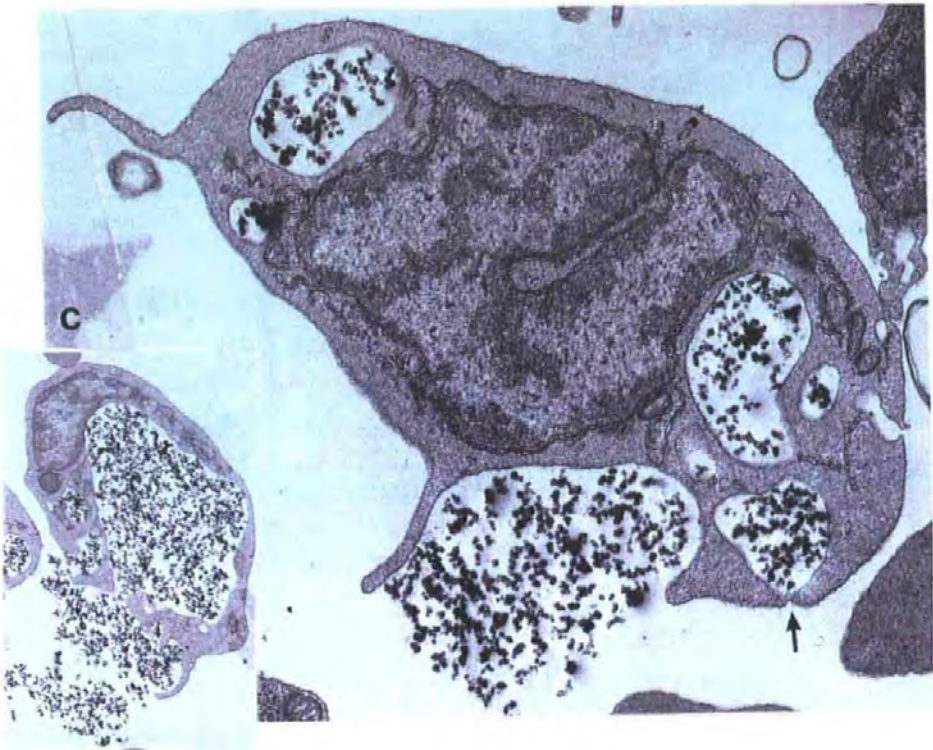
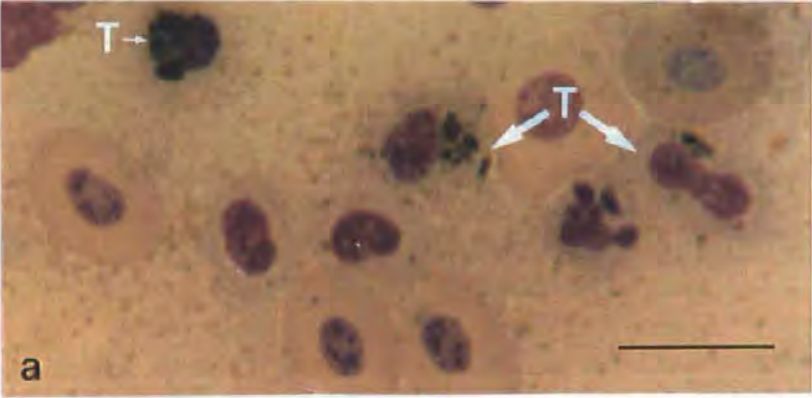
#### **3.3.3.1 Light Microscopy**

Lymphocytes were characterised by a large nucleus which was usually indented. The nucleus stained less intensely than that of the thrombocyte, occupying almost the entire cell (Figure 7a), resulting in a high N:C ratio of 0.83. Numerous fine pseudopodia of various lengths extended from the narrow rim of basophilic, agranular cytoplasm surrounding the nucleus. Small and large lymphocytes were observed and classified by their diameters and relative amounts of cytoplasm (Figure 7b). The latter were only rarely encountered constituting just 1% of the lymphocyte population. The high N:C

**Figure 6**     *In vitro* phagocytosis of colloidal carbon by thrombocytes.

- a**     Light micrograph showing thrombocytes (T) containing internalised carbon particles. (MGG). (Bar represents 10 $\mu$ m)
  
- b**     Electron micrograph showing internalisation of colloidal carbon (C) by a thrombocyte at 12°C. (Mag x 10,050).
  
- c**     Pseudopodal processes of thrombocytes encircling carbon particles at 22°C. Arrow indicates phagosome nearing completion of formation. (Mag x 9,740).  
  
        Inset: A very heavily laden thrombocyte with the majority of carbon contained within a single large vesicle.  
                (Mag x 6,500).



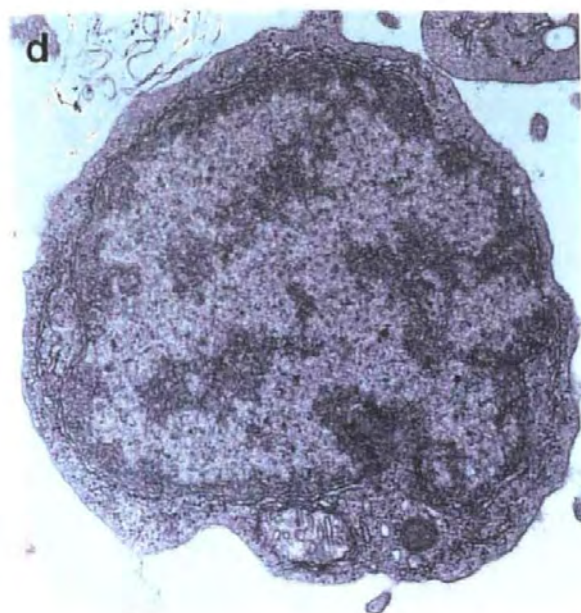
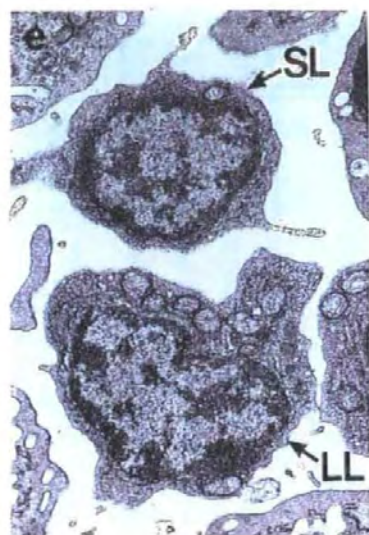
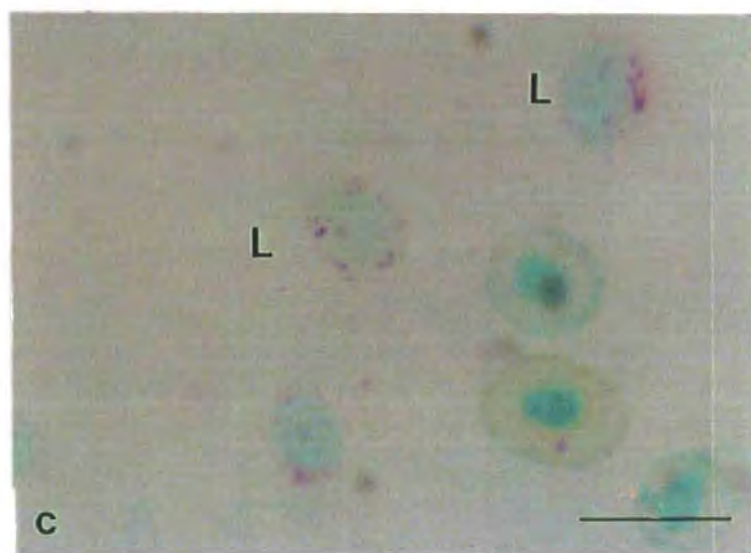
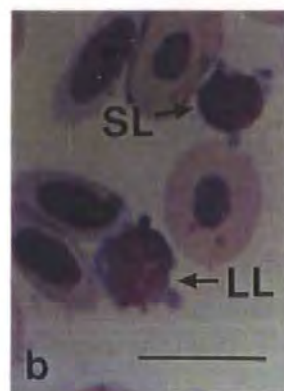
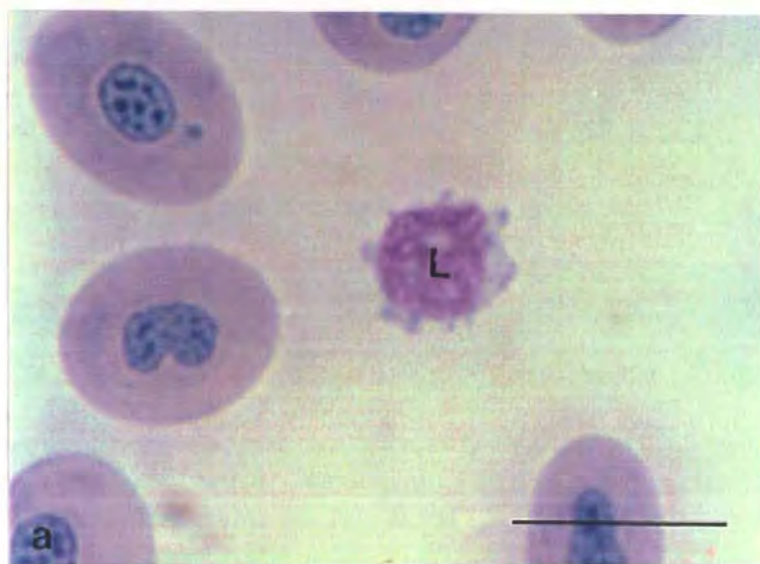


**Figure 7      Lymphocytes**

- a                    MGG staining of a lymphocyte (L).**
- b                    Large (LL) and small (SL) lymphocytes. (MGG)**
- c                    Acid phosphatase staining showing positive staining in lymphocytes (L).**

**(Bars represent 10µm)**

- d                    Electron micrograph of a lymphocyte. (Mag x 12,500).**
- e                    Electron micrograph of a small (SL) and large (LL) lymphocyte, the latter containing a large number of mitochondria. (Mag x 4,850).**





ratio was reduced slightly in larger lymphocytes which contained a greater proportion of cytoplasm (Table 1).

### **3.3.3.2 Histochemistry**

The majority of lymphocytes stained positively for acid phosphatase (Figure 7c) with particulate staining appearing in the cytoplasm.

Other histochemical tests employed to detect lipid, glycogen, alkaline phosphatase and non-specific esterase activity were all negative.

### **3.3.3.3 Electron Microscopy**

The centrally positioned round to oval nucleus was deeply indented, appearing in some sections to be composed of two nuclear portions. Lymphocytes contained dense, radially arranged nuclear chromatin enclosing the inter-chromatin material and a nucleolus was occasionally seen. These frequently observed cells resembled transversely sectioned thrombocytes.

The nucleus of small lymphocytes was surrounded by a narrow band of sparse agranular cytoplasm surrounding the nucleus often exhibiting an irregular outline and being characterised by occasional microvilli (Figure 7d). Numerous free ribosomes were scattered throughout the cytoplasm. Large, elongated mitochondria exhibiting prominent tubular cristae occurred frequently, often accumulating in one region of the cell. Up to eight mitochondria were seen in one lymphocyte section (Figure 7e). Large lymphocytes were characterised by their greater amounts of cytoplasm and larger mitochondria. Distinct profiles of rough endoplasmic reticulum (visualised as basophilic areas at the LM level) along with occasional Golgi, made up the few cytoplasmic organelles seen.

#### **3.3.3.4 Phagocytosis**

No carbon particles were observed within the cytoplasm of this cell type.

### **3.3.4 GRANULOCYTES**

#### **3.3.4.1 Light Microscopy**

Spherical granulocytes contained an eccentrically positioned, rounded nucleus, occupying between a quarter and a third of the cell (Figure 8a). The darkly staining nucleus, ranged from round or oval to deeply indented. Occasionally a segmental form was seen with a maximum of two lobes (Figure 8b). The cytoplasm of these large round cells contained granules which were unstained with MGG.

#### **3.3.4.2 Histochemistry**

PAS staining resulted in a strong, dense, granular positivity throughout the cytoplasm (Figure 8c). The most intense reaction was observed in granulocytes with lowest N:C ratios. Pre-digestion with salivary amylase resulted in these cell types being negative, indicating the presence of glycogen. Discrete, scattered granules of strong sudanophilia were observed following staining with Sudan black B (Figure 8d). Acid phosphatase (Figure 8e) and alkaline phosphatase (Figure 8f) activities were also detected.

The granulocyte type found, exhibited morphological and histochemical properties of the mammalian neutrophil. No eosinophilic or basophilic granulocytes were observed.

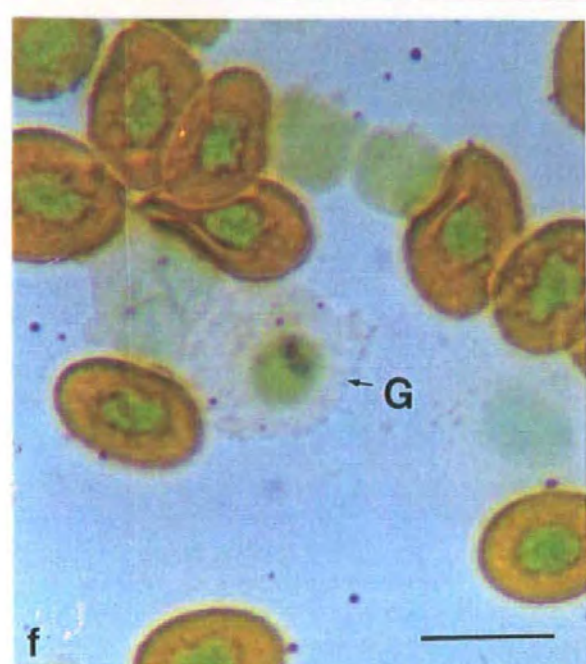
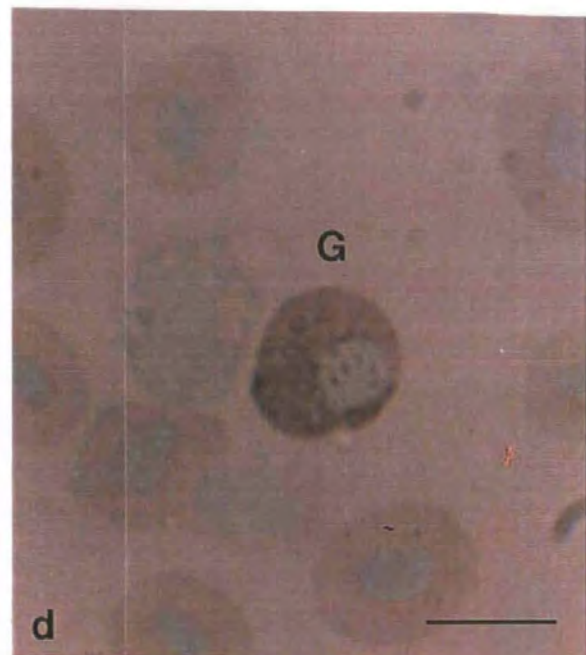
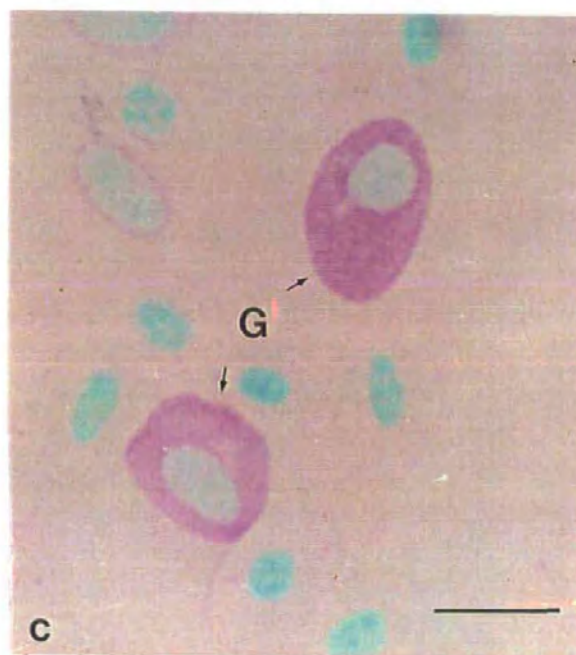
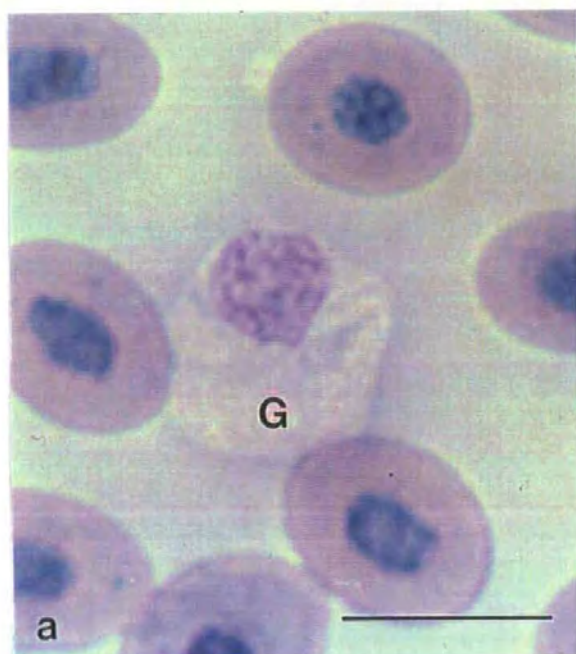
#### **3.3.4.3 Electron Microscopy**

The nucleus was eccentrically positioned and characterised by a moderately electron dense peripheral layer of chromatin and dense areas of interchromatin. Polymorphic forms were rarely seen.

**Figure 8      Granulocytes**

- a            MGG staining of a granulocyte (G).**
- b            Granulocyte (G) with a bilobed nucleus. (MGG).**
- c            PAS positivity in granulocytes (G).**
- d            Sudan black B staining of a granulocyte.**
- e            Acid phosphatase showing positive staining of granulocytes (G).**
- f            Alkaline phosphatase showing positive staining of granulocytes (G).**

**(Bars represent 10µm)**



The relatively smooth cytoplasmic margin showed few surface projections. Most notable features of granulocyte cytoplasm were the presence of numerous, elongate cytoplasmic granules (Figure 9a). Membrane bound, rod-shaped granules were rounded or elliptical in section, ranging in size from 0.5 x 0.2 $\mu$ m to 1.0 x 0.3 $\mu$ m. They contained an electron dense matrix of varying intensity, separated from the limiting membrane by an electron lucent zone seen as a clear halo. Some granules were seen to contain fibrillar inclusions. Numerous aggregates of irregularly shaped granules 20 - 30nm in diameter were also seen within the cytoplasm.

Many parallel arrays of rough endoplasmic reticulum were observed localised close to the nucleus or in the nuclear cleft (Figure 9b).

Rounded or elongate mitochondria scattered throughout the cytoplasm, were fewer in number than observed in lymphocytes. Golgi bodies comprising a few flattened sacs were only occasionally encountered. The cytoplasm of immature cells with their non-lobate centrally positioned nucleus contained a high proportion of rER and only a few small granules. More mature cells possessed a lobate nucleus, with smaller amounts of rER. However their granules were larger in size and more numerous.

#### **3.3.4.4 Phagocytosis**

No carbon particles were observed within the cytoplasm of these cells at LM or EM level at either temperature.

### **3.3.5 MONOCYTES**

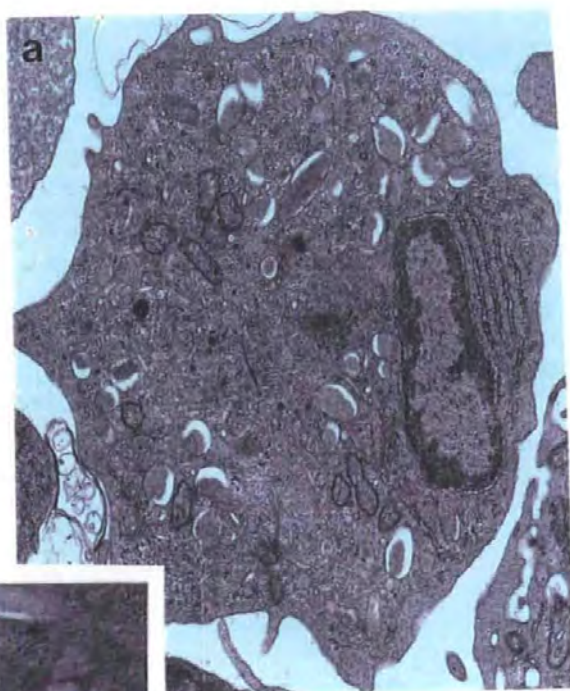
#### **3.3.5.1 Light Microscopy**

Monocytes were the largest and most variably shaped of the peripheral blood leucocytes. The large spherical or indented nucleus of this rarely encountered leucocyte occupied half the cell, occasionally exhibiting the classical "horse-shoe" shape. The relatively abundant basophilic cytoplasm



**Figure 9      Ultrastructure of the granulocyte.**

- a              Granulocyte. (Mag x 7,700).**  
**Inset: Detail of rod-shaped cytoplasmic granules.**  
**(Mag x 15,200).**
- b              Parallel rays of endoplasmic reticulum within the nuclear cleft of**  
**a granulocyte. (Mag x 4,450).**



was often vacuolated (Figure 10a). Monocytes adhered to glass surfaces with a tendency to spread during *in vitro* observations.

#### **3.3.5.2 Histochemistry**

Alkaline phosphatase (Figure 10b) and a diffuse acid phosphatase (Figure 10c) activity was detected. Scanty, scattered non-specific esterase positivity of monocytes was also observed (Figure 10d).

Monocytes were unstaining with Sudan black B and Periodic acid Schiff (PAS) stains.

#### **3.3.5.3 Electron Microscopy**

Dense chromatin was localised around the periphery of the pale staining nucleus. Abundant agranular cytoplasm was drawn into irregular and lengthy pseudopodia and microvilli extending from the cell surface. Cytoplasm rich in large mitochondria with distinct cristae was often a predominant feature. Occasional electron lucent vesicles appeared to contain cellular debris and amorphous material (Figure 11a). Long profiles of rER and numerous scattered lysosomes were also evident.

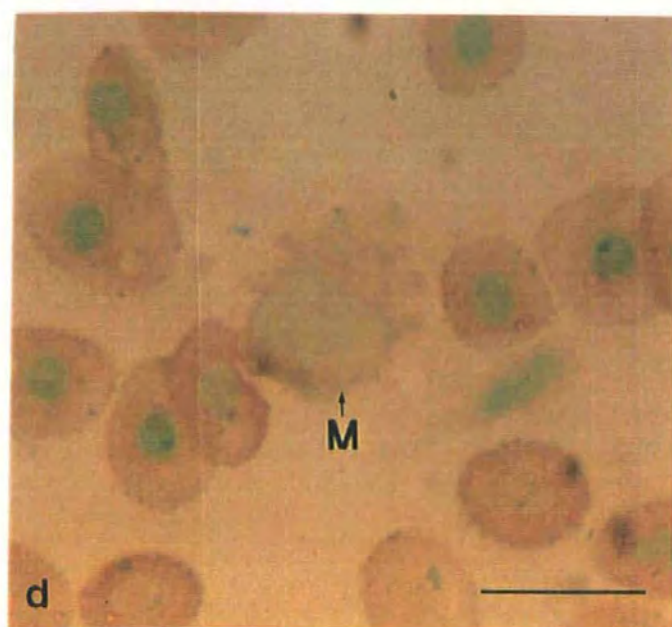
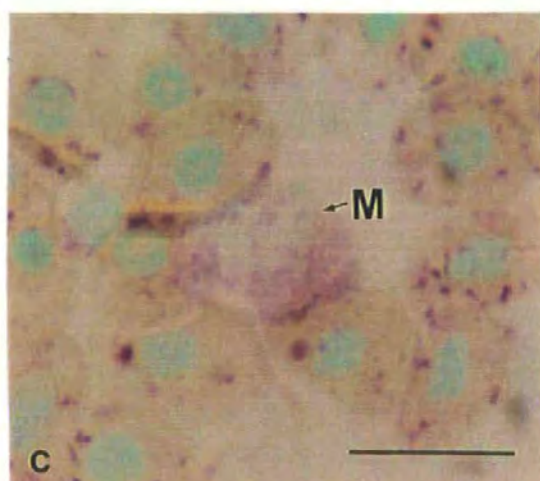
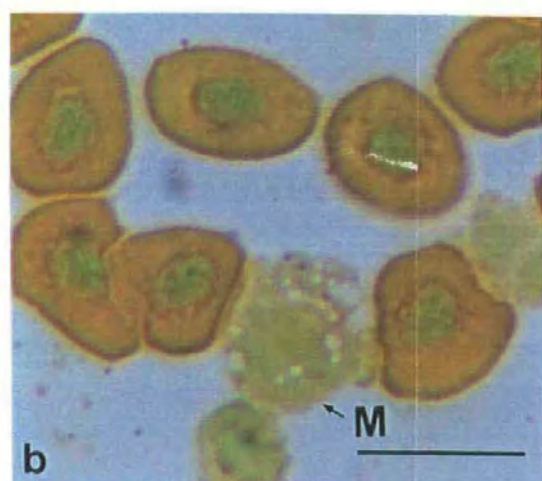
#### **3.3.5.4 Phagocytosis**

Some carbon particles were observed within the cytoplasm of this cell type at LM level (Figure 11b) at both temperatures.

**Figure 10    Monocytes**

- a            MGG stained monocyte (M).**
  
- b            Alkaline phosphatase staining showing positively stained monocyte (M).**
  
- c            Acid phosphatase staining showing positively stained monocyte (M).**
  
- d            Non-specific esterase staining showing positively stained monocyte (M).**

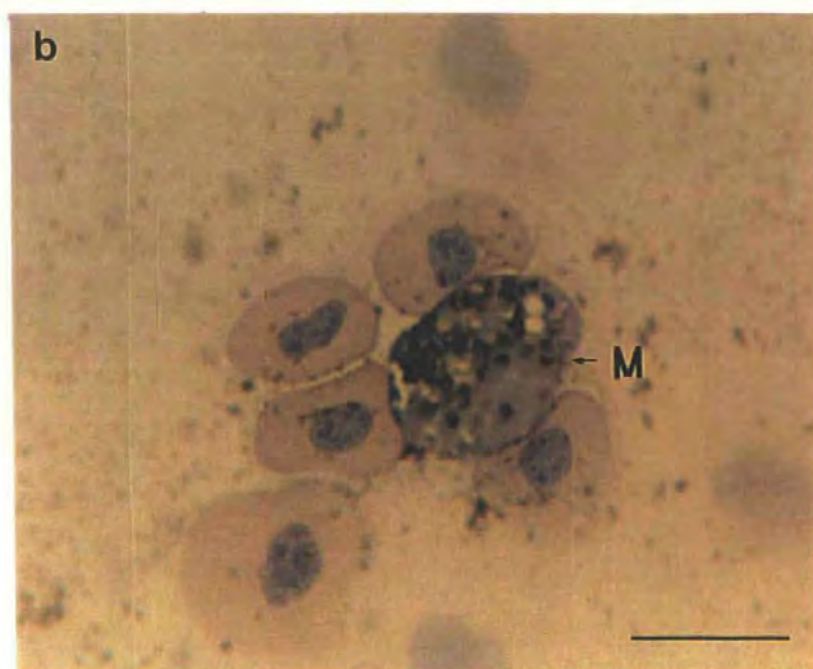
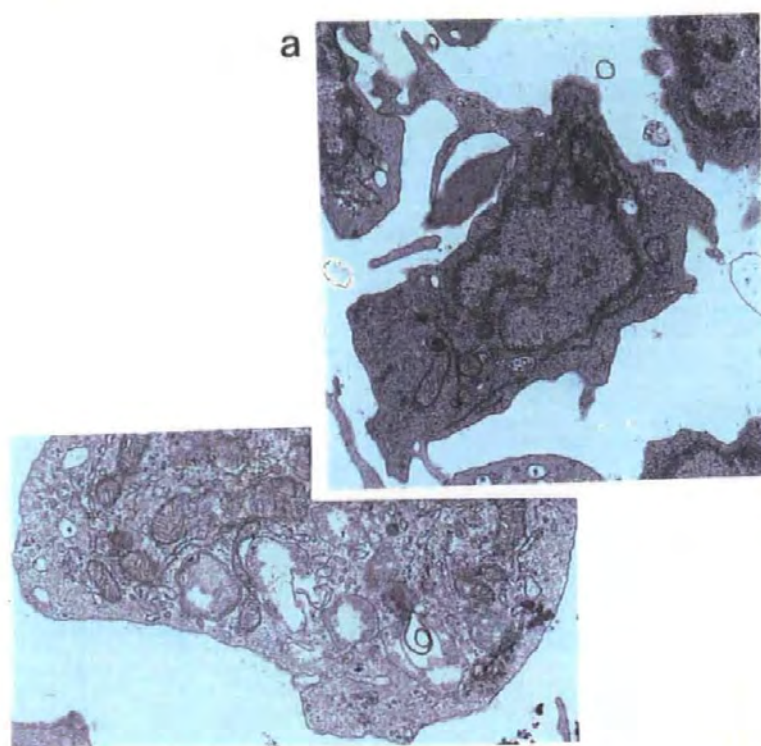
**(Bars represent 10µm)**



**Figure 11    Monocyte ultrastructure and phagocytosis.**

- a**            Electron micrograph of a monocyte showing extrusion of pseudopodia. (Mag x 4,600).  
Inset: Cytoplasmic inclusion of apparent cellular debris and amorphous material. (Mag x 9,560).
- b**            Light micrograph showing *in vitro* uptake of colloidal carbon by a monocyte (M) at 22°C. (MGG). (Bar represents 10µm).





## CHAPTER 3

### 3.4 DISCUSSION

Well prepared peripheral blood films stained with Romanowsky stains provided a basis for leucocyte identification in this study. May Grunwald and Giemsa was the most useful, being easily reproducible and providing good nuclear and cytoplasmic differentiation. Turbot leucocytes were found to have distinct morphological similarities to the leucocytes of mammals and other fish. They consisted of four cell types; thrombocytes, lymphocytes, neutrophil-like granulocytes and monocytes. Relative sizes of turbot leucocytes follow the general vertebrate pattern (if thrombocytes are excluded): monocyte > granulocyte > large lymphocyte > small lymphocyte. If such cells which morphologically resembled those in mammals, were in fact functional equivalents, then they would be expected to respond similarly to histochemical tests.

Total erythrocyte and leucocyte counts recorded for the turbot fall within the ranges found in other fish species. Haematocrit values for the turbot of 22.7% fall just outside the ranges of 25% - 50% given for other species. As results can be affected by erythrocyte swelling which occurs in anaesthetised or stressed fish (Ross, McKinney and Coutts, 1986), fish were sampled without the use of an anaesthetic. Snieszko (1960) suggested the usefulness of the haematocrit to check anaemia in the field with microcapillary tubes reducing the need for several millilitres of blood which is often not available from small fish. Ellis (1976) found leucocyte numbers in the plaice, *Pleuronectes platessa*, to be very variable. Large variations in red and white cell numbers have been attributed to stress, including handling (Casillas and Smith, 1977), season, and anaesthesia.



Leucocyte numbers in fish are generally higher than those of mammals. This is due, in part, to the inclusion of thrombocytes in counts of fish, whereas platelets are excluded in mammalian leucocyte counts. However, this alone does not account for the higher numbers of leucocytes observed in fish. In the present study stress during capture and handling was minimised as it resulted in rapid clotting of blood samples having a huge effect on haematological parameters. Haemolysis of cells was also avoided by ensuring only thin blood smears, produced and dried as rapidly as possible were used. Since leucocytes distort during the preparation and drying of smears, cell sizes in such preparations can only be compared directly with those given for other smears and not in live preparations.

Initially, the occurrence of damaged leucocytes in blood film preparations was a problem in the present study. To overcome this, only apparently healthy fish were used and blood was always collected with the minimum of stress. Damaged cells which appear as amorphous, purple areas in blood smears have been named "smudge cells" by Williams and Warner (1976) who suggest that these are associated with the disruption of erythrocytes. Such cells were found to be numerous here in badly prepared thick smears, in blood sampled from stressed fish and when certain anticoagulants were used, most notably heparin and EDTA. In this study TSC was found to be the most effective anticoagulant, causing least cell lysis. It would appear that there is considerable inter-species variation in the effectiveness of different anticoagulants. Heparin has been reported to cause marked staining differences in cells of the plaice (Ellis, 1977). The effects of anticoagulants on the leucocytes of the blenny (*Blennius pholis*) were assessed by Mainwaring and Rowley (1985) who found heparin to be most suitable. They reported that EDTA was adequate but TSC failed to prevent coagulation at any concentration tested and suggested that this may be due to the high osmolarity and pH of TSC. That heparin and EDTA have lower osmolarities and pH may account for their lack of reliability in the present

study but their apparent suitability as anticoagulants in fresh water species. These findings suggest that certain anticoagulants are more effective for different fish species and a range should be assessed prior to any haematological studies being conducted.

The variability in turbot thrombocyte shape observed in the present study is supported by the findings of many authors including Ellis (1977) who categorised these different forms into 4 specific types namely spiked, spindle, ovoid and lone nucleus. However, shape has been attributed to a variety of causes including artefact, clotting function and maturity. Observations of live thrombocytes here and in the plaice (Wardle, 1971) suggest that these cells are capable of a high degree of movement including cell extension and shortening, twisting and waving of spindle filaments resulting in many different shapes being seen. The lone nucleus form was rarely seen in well prepared blood films but has been commonly reported in stressed fish, suggesting that they are a function of the blood clotting mechanism. Stress, known to cause increased clotting rates, would result in a high proportion of thrombocytes with denuded nuclei.

Findings here, that the different shapes of this cell type are not artifactual, support those of Volynkin (1983) who suggested that classification of thrombocytes into separate types is artificial after describing thrombocyte shapes transitional between all forms. Mahajan and Dheer (1979) state that normal thrombocytes are oval but are rarely seen due to changes in the cell which occur rapidly as a result of the clotting function. Gardner and Yevich (1969) however, explain this shape variability in terms of cell maturity and proposed the study of live rather than fixed preparations.

Ultrastructural findings of pseudopodia, organelle free areas and the presence of microtubules suggests turbot thrombocytes are capable of movement. The presence of large numbers of microtubules below the plasma membrane,

occasionally running the length of the cell, has been equated with motility. In mature cells, microtubules aggregate into a marginal band. Microvilli reported in carp, *Cyprinus carpio*, (Cenini, 1984) and plaice (Ferguson, 1976) were only seen in this study in cells phagocytosing carbon particles. Boomker (1980) described the reactive stages during clotting when the cell membrane is clearly visible, cytoplasm vacuolated with the nucleus rounding up and becoming pyknotic. *In vitro* observations here further suggest that the cytoplasm is reduced and in the final stages, very small cells with intensely staining nuclei are surrounded by fine radiating fibrin threads.

In the present study, thrombocytes represented 52% of circulating leucocytes in the turbot. However, Quentel and Obach (1992) found that this cell type made up only 20.2% of total blood leucocytes in the same species. A possible explanation for this discrepancy may lie in the marked fragility of this cell type. Since the latter authors did not use an anticoagulant when collecting blood samples, it may be feasible that increased lysis occurred as a result. However, enormous variation in thrombocyte number has been reported ranging from 58% in *Chana punctatus* (Mahajan and Dheer, 1979) to just 1.8% in rainbow trout, *Onchorhynchus mykiss*, (McCarthy, Stevenson and Roberts, 1973). Differential counts made by Saunders (1966) on 121 species showed thrombocytes to be the most prevalent leucocyte type, followed by small lymphocytes. Variations have even been reported within species. Weinreb and Weinreb (1969) identified 3-13% of goldfish leucocytes as thrombocytes, whereas Gardner and Yevitch (1969) found 82-95% in the same species. Difficulties in differentiating between thrombocytes and lymphocytes may explain such discrepancies. Variation in counts may be due to clotting and smears should be discarded whenever this has obviously occurred. Indeed, Srivasta (1969, cited Mainwaring and Rowley, 1985) stated that clotting rate is a function of the number of thrombocytes present. Fragility of leucocytes has often been cited as a difficulty in such studies (Ellis, 1976; Ferguson, 1976; Weinreb, 1963).

Thrombocyte nuclei can easily be denuded of cytoplasm during smear preparation resulting in their misidentification as lymphocytes. Problems in distinguishing between rounded thrombocytes and small lymphocytes have been frequently reported (Ellis, 1977; Hines and Yashov, 1970 cited Ellis, 1977) and may result in unrealistically high counts of one or other cell type. The great similarity led Boomker (1980) to suggest that the thrombocyte was actually a small lymphocyte specialised to fulfil a specific function.

At the LM level and, if sectioned transversely, at the EM level turbid thrombocytes and lymphocytes appear similar. In this study they were categorised by the more basophilic undulating cytoplasm of the lymphocyte when compared to the very discrete, pale staining cytoplasm of the thrombocyte. Such cells were difficult to differentiate in poor smears due to distortion and cell clumping. The more intensely staining nucleus of the thrombocyte when compared to the lymphocyte is a finding reported for other fish species (Williams and Warner, 1976). Histochemical differences, the presence of microtubules and N:C ratios also helped differentiation between these cell types.

Thrombocytes appear more prevalent in marine rather than in fresh water perciform fish. Volynkin (1983) stated that thrombocytes of fresh water fish have partially lost their role in evolution resulting in them being present in reduced numbers.

Differing from their mammalian platelet counterparts, the thrombocytes of fish are much larger and nucleated. It has been suggested by Ellis (1977) that, due to their prevalence in large numbers in the plaice spleen, thrombocytes are produced in splenic tissue. It is known that under stress, fish are capable of contracting the spleen effecting an increase in the volume of circulating blood and thereby releasing large numbers of thrombocytes into the circulation. This may account for some of the variations in thrombocyte

numbers reported, underlining the fact that stress should be minimised when undertaking such measurements and its effects should not be underestimated.

At the ultrastructural level turbot thrombocytes resembled mammalian platelets in that they displayed a series of vacuoles within the cytoplasm. Ferguson (1976) has suggested that these may be analogous with the surface connecting system seen in the mammalian platelet. Although many times smaller, platelets also possess a large number of vesicles with mitochondria and granular inclusions in the cytoplasm. However, the large nucleus so obvious in fish thrombocytes is lacking. The similarity is most obvious when observing thrombocytes sectioned through the cytoplasm in such a way as to avoid the nucleus. Fish thrombocytes are richer in mitochondria than mammalian platelets, perhaps indicating a more active cell type.

The cytoplasm of turbot thrombocytes did not appear granular in the present study. This is in contrast to the findings of Boomker (1980) who reported a granular cytoplasm in catfish thrombocytes. A single granule (lysosome) has been reported in the thrombocytes of the Arctic icefish (Barber, Westermann and White, 1981) and the plaice by Ellis (1977) at the base of spiked thrombocytes. A specific acidophilic granule has been reported by Boomker (1980). Numerous fine and coarse granules of PAS positivity, indicating the presence of polysaccharides, including glycogen, were scattered throughout the cytoplasm of turbot thrombocytes. Thrombocytes of lower vertebrates also have a high glycogen content (Lewis and Shiraleana, 1962, cited Boomker, 1980), a feature also observed in many fish studied by Hine *et al* (1987), in the plaice (Ellis, 1976) and the dogfish, *Scyliorhinus canicula*, (Parish *et al*, 1986). In contrast, Blaxhall and Daisley (1973) found that thrombocytes of the brown trout, *Salmo trutta*, displayed no PAS activity. Sudan black B staining of turbot thrombocytes was negative suggesting a lack of lipid, as found for the cichlid *Oreochromis mossambicus* (Doggett, Wrathmell and Harris, 1987). The acid phosphatase activity found here has

also been reported in mammalian platelets and in the icefish (Barber and Westermann, 1981). Observation of this hydrolytic digestive enzyme is indicative of the presence of lysosomes, a feature compatible with a phagocytic capability (Barber and Westermann, 1981).

The uptake of carbon by mammalian platelets has been reported (Van Atken *et al*, 1970 - cited Ferguson, 1976). Fish thrombocytes also have an ability to phagocytose foreign material. Evidence of *in vitro* and *in vivo* phagocytosis by thrombocytes of different fish species exists. Results presented here are supported by the findings of Ferguson (1976) and Morrow and Pulsford (1980). Suzuki (1984) reported weak *in vitro* phagocytic activity of thrombocytes from the rodfish, *Sebastes schlegeli*, exposed to bacteria, as did Kusada and Ikeda (1987) in the eel (*Anguilla anguilla*). However, in contrast, Hyder, Cayer and Petty (1983) found no evidence of phagocytosis in thrombocytes of the nurse shark, *Ginglymostoma cirratum*. Ellis (1976) suggested that the presence of foreign material within this cell type is due to mechanical entrapment, rather than active phagocytosis. However, the rapid rate at which carbon appeared within this cell type following *in vitro* exposure and the observation of extended pseudopodia at the EM level suggest that uptake by turbot thrombocytes is indeed an active process.

The thrombocyte structure of many fish species is relatively consistent, appearing as morphological and functional nucleated homologues of the human blood platelets, sharing their function in blood clotting processes (Gardner and Yevich, 1969) as described by Wardle (1971) and similar to thrombocytes of birds.

Turbot lymphocytes appear structurally similar to mammalian lymphocytes, with the largest nuclear : cytoplasmic (N:C) ratio of all leucocyte types observed. They follow the typical vertebrate pattern with N:C ratios of 0.78 and 0.83 and display few cytoplasmic organelles. They are present in larger

numbers in fish than in man (Ellis, 1977) as confirmed by this study. Lymphocytes constitute a population of cells within which a continuum of sizes has been reported (Roubal, 1986; Elarifi, 1982; Barber and Westermann, 1981). This has often been arbitrarily divided up into large and small lymphocytes. In the turbot their sizes range in diameter from 6µm to 9 µm, with similar N:C ratios of 0.83 and 0.78. In common with other fish species, larger lymphocytes, often confused with monocytes, were differentiated by their more abundant cytoplasm (lacking phagosomes) and somewhat reduced N:C ratio. In their study of the haematology of the porcupine fish (*Diodon hystrix*), Radhakrishnan, Stephen and Balakrishnan (1976) reported large numbers of lymphocytes (61% of leucocytes) and neutrophils (12%) but found no thrombocytes. Drawings and reports appear to suggest some misidentification with thrombocyte - like cells being called small and medium lymphocytes or neutrophils. Reports of lymphocyte counts as high as 91.2% exist for the lake trout, *Salmo namaycush* (Lieb, Slane and Wilber, 1953). Observation of fine structure and use of histochemistry were not employed by these authors and may have assisted identification.

Lymphocyte cytoplasm was basophilic, reflecting the presence of a large number of ribosomes which bear a negative charge and therefore attract basic dyes. Ultrastructural observations confirmed the presence of large quantities of rough endoplasmic reticulum.

As in other fish species, lymphocytes reacted with few histochemical stains. Acid phosphatase activity, detected in most turbot lymphocytes, is present in the lysosomes of many haematopoietic cells, being more intense in mammalian T-cells than B-cells.

Turbot lymphocytes displayed no phagocytic activity here which is supported by the findings in many fish species including the plaice (Ellis, 1977).

Although several reports of phagocytosis by fish lymphocytes do exist (Weinreb and Weinreb, 1969; Klontz, 1972), this may reflect difficulties in differentiating between lymphocytes and thrombocytes. Bielek (1979) suggested this might be overcome by employing electron microscopy to visualise cytoplasmic vesicles and microtubules. It may also account for some of the huge discrepancies in lymphocyte numbers ranging from 91% (Lieb, Slane and Wilber (1953), cited Boomker, 1981) to 2-5% (Mahajan and Dheer, 1979), since it seems unlikely that interspecific variation alone can explain such large differences.

In the present study, turbot granulocytes measured  $11\mu\text{m}$  in diameter and represented 5.6% of the leucocyte population. This is similar to the findings of Ellis (1976) for plaice granulocytes which made up 5-10% of blood leucocytes but at variance with the findings of Quentel and Obach (1992) who identified 30.2% of turbot leucocytes as granulocytes. Such a large discrepancy with the results presented here is difficult to explain but may reflect an undetected stimulated immune state. Granulocytes represent a smaller proportion of leucocytes in fish than in mammals where they are the most abundant leucocyte type (>70%). This discrepancy may be related to the phagocytic role of the thrombocyte in fish, a function normally associated with the granulocytes in mammals.

Only one granulocyte type was found in the present study. It most closely resembled the mammalian neutrophil and similar findings have been reported in the plaice (Ellis, 1976; Ferguson, 1976), bream (Roubal, 1986) and catfish (Boomker, 1981). Ellis (1977) stated that this granulocyte type was the most abundant encountered in teleost species. The three types of human granulocyte; neutrophils, basophils and eosinophils which have different staining affinities for basic and acidic dyes (Erich, 1880, cited Lester and Desser, 1975) have all been reported in fish. Cenini (1984) recorded all three granulocyte types in the carp. However, the literature pertaining to fish



granulocytes is very complex and full of anomalies. Eosinophils have been reported in the sparid *Acanthopagius australis* (Roubal, 1986), the dogfish (Morrow and Pulsford, 1980) and the rainbow trout (Ezeasor and Stokoe, 1980). Basophils have been reported in the carp (Suzuki, 1986), the channel catfish, *Ictalurus punctatus* (Williams and Warner, 1976) and the goldfish, *Carrasius auratus* (Weinreb, 1963). However, these findings were questioned by Barber and Westermann (1978) who were unable to detect any such cell in the 58 fish species they examined. Such differences may reflect a limitation of staining procedures, the occurrence of such cells in very small numbers or only during certain states of health. Neutrophil equivalents in fish have also been referred to as heterophils. However, the terms heterophil and neutrophil have been regarded as synonymous by Barber and Westermann (1978) but separate by Saunders (1966).

Turbot granulocytes were characterised by nuclear morphology and tinctorial properties of the cytoplasmic granules which were unstained with acid or basic dyes. Although the granulocyte nucleus was usually rounded, occasionally bilobed forms were observed. However, whether these cells should be regarded as polymorphonuclear leucocytes (PMNLs) is questionable as the segmentation of up to 4 lobes as recorded for mammalian PMN granulocytes were never seen. Ultrastructurally the large cytoplasmic granules observed within the turbot granulocyte were of similar structure to those described in neutrophils of the plaice (Ferguson, 1976), eel, *Anguilla australis* (Hine, Wain, Boustead and Dunlop, 1986), and dogfish (Doggett, 1989).

Intense sudanophilia of turbot granulocytes, indicative of a high lipid content, has also been reported in plaice (Ellis, 1976) and brown trout (Blaxhall and Daisley, 1973; Roubal, 1986) neutrophils. However, dogfish (Parish *et al*, 1986), lamprey, *Lampetra fluviatilis* (Kelenyi and Larsen, 1976) and white sucker, *Catostomus commersonnii* (Barber and Westermann, 1975)

neutrophils were sudan black B negative. As some interspecific variations exist in the histochemical reactions of mammalian neutrophils, one might expect some differences between fish species.

In the turbot, neutrophil-like granulocytes were PAS positive. Such activity has also been recorded in the neutrophils of many species (Blaxhall and Daisley, 1973; Ellis, 1976; Barber and Westermann, 1978; Roubal, 1986) as in man. The PAS reaction was negative in turbot following pretreatment with salivary amylase, supporting the findings of Cannon *et al* (1980) in the catfish and Ellis (1976) in the plaice and indicating the presence of glycogen. All developmental stages of turbot granulocytes were PAS positive and, as in mammalian studies, most intense reactions were observed in the oldest cells, suggesting the synthesis of glycogen proceeds with age. Distinct cytoplasmic particles of glycogen (thought to be a potential carbohydrate energy store for the cell) also increase in number as the cell matures.

Turbot granulocytes produced a Sudan black B staining pattern consistent with mammalian neutrophils with the cytoplasm packed with positively stained granules. Sudan black B staining of granules and cytoplasm has been used to differentiate between granulocyte types in mammals. In eosinophils, granules remain unstained with the cytoplasm reacting positively and in basophils, both the granules and cytoplasm remain unstained.

The alkaline phosphatase activity within the granulocyte cytoplasm reported here has also been described by Ellis (1976) in a few plaice neutrophils. Common features of the majority of blood neutrophils of vertebrates are PAS and alkaline phosphatase positivity. Alkaline phosphatase activity was found within the granules of goldfish (Garavini and Martelli, 1981) and rainbow trout granulocytes (Ezeasor and Stokoe, 1980).

Although the appearance of fish neutrophils is similar to those of mammals, their function is uncertain and there is some contention as to whether they are phagocytic. Ultrastructural features of turbot granulocytes including the presence of pseudopodia and some organelle-free areas indicate they are capable of movement and possess well developed secretory apparatus. The acid phosphatase activity seen in turbot granulocytes is normally demonstrable in mammalian phagocytic cells and was also described by Ellis (1976) in the plaice and by Parish *et al* (1986) in the dogfish. Its occurrence in fish neutrophils suggests the presence of lysosomes and hence a phagocytic capability. Ezeasor and Stokoe (1980) failed to find evidence for hydrolases (including acid phosphatase) in rainbow trout. The possession of such lysosomal enzymes by thrombocytes and monocytes, both phagocytic in the turbot, might suggest that granulocytes have the same capacity.

The lack of phagocytic activity seen during *in vitro* studies may reflect sub-optimal experimental conditions. Since it is known that some cells are phagocytic towards bacteria whilst their uptake of carbon particles is limited, turbot granulocytes may have a phagocytic capacity for alternative foreign materials. Such activity may also occur *in vivo* but remain undetectable *in vitro*. Contention exists within the literature concerning phagocytic activity of fish neutrophils even within the same species. Phagocytosis was reported in plaice by MacArthur and Fletcher (1985) and Ferguson (1976) but not by Ellis (1976). Finn and Neilson (1971) found this cell type phagocytic in rainbow trout opposing the findings of Klontz (1972). In his review on fish granulocytes, Hine (1992) concluded that the degree to which teleost neutrophils phagocytose pathogens and particles is variable but that in most species they are capable of phagocytosis *in vitro* and *in vivo*. However, fish neutrophil sub-populations may differ in their phagocytic capability (eg during inflammation) with variability between individual fish as well as between species. Further ontogenetic and functional studies, therefore, are required to unravel the identification and nomenclature of fish granulocytes.

Turbot monocytes were the largest and most rarely encountered cell type in the present study. Morphologically they resembled descriptions given for other fish (Ellis, 1976; Ferguson, 1976; Elarifi, 1982; Cenini, 1984). These motile cells exhibit pseudopodia, abundant, slightly basophilic cytoplasm, an indented nucleus and their rER, Golgi complex, vesicles and cytoplasmic granules resemble those of monocytes of other teleosts. Staining was similar to that of other fish species, exhibiting weak non specific esterase activity as reported in the lamprey, *Lampetra fluviatilis* (Kelenyi and Larsen, 1976). A more intense staining has been reported in the dogfish by Parish *et al* (1986) and mammalian monocytes. Turbot monocytes stained positively for acid and alkaline phosphatase, findings also reported by Ellis (1976) who recorded similar reactions in plaice monocytes.

Turbot monocytes represent an apparently larger proportion of the total leucocyte population when compared with the plaice (Ellis, 1976). It has been suggested that this cell type is absent in the goldfish (Weinreb and Weinreb, 1969) and brown trout (Blaxhall and Daisley, 1973). However, confusion of monocytes with large lymphocytes has been suggested to account for this discrepancy by Roubal (1986).

The observation of phagocytosis by turbot monocytes has previously been reported in other species for carbon (Ellis, 1976; Ferguson, 1976; McKinney, Smith, Haynes and Sigel, 1977), yeast (Hyder *et al*, 1983) and for bacteria (Suzuki, 1984). McKinney *et al* (1977) reported the major phagocytic cell type in the gar, *Lepisosteus platyrhincus*, to be the monocyte rather than the granulocyte, as is the case in mammals. Phagosomes containing internalised cellular material and the presence of the hydrolytic enzymes, non-specific esterase and acid phosphatase seen here in turbot monocytes are indicative of a phagocytic capability. These findings suggest an endocytic function for fish monocytes including the removal of foreign material from the bloodstream

and therefore, assessment of phagocytosis during identification studies may help to differentiate between monocytes and large lymphocytes.

The leucocytes of turbot show distinct similarities to those of higher vertebrates and other fish species, especially the plaice. Results from histochemical characterisation and morphological studies suggests that they are functionally equivalent cells and in the case of turbot, the use of mammalian nomenclature in their description appears justified.

## **CHAPTER 4**

### **TISSUE COMPONENTS OF THE LYMPHOID SYSTEM**

#### **4.1 INTRODUCTION**

The function of the immune system is to provide protection against invading micro-organisms such as viruses, bacteria, fungi, and parasites. Leucocytes, which form the basis of this defence, have been identified in Chapter 3. They are distributed throughout the body in either discrete lymphoid organs including the thymus, kidney and spleen, or in more diffuse tissue accumulations which tend to occur in areas where pathogens may gain direct access from the external environment. Such accumulations include the gut associated lymphoid tissue (GALT) (Davina, Rijkers, Rombout, Timmermans and van Muiswinkel, 1980) and the scattered lymphoid cells seen in the dermis of the skin (Peleteiro and Richards, 1985) and in the gills (Roberts, 1989). Organisation of the tissue and organs in this way enables them to maximise their effectiveness.

Evolutionary advances have been correlated with an increased diversity and efficiency of the immune response coupled with increased complexity and organisation of lymphoid tissues (Pitchappan, 1980). In mammals, bone marrow is important in the formation of stem cells. However, fish have no bone marrow. They also lack lymph nodes and produce red and white cells within haemopoietic tissues found outside the skeleton (Fänge, 1994). They possess a whole range of haemopoietic tissues resembling those found in mammals and which maintain a balance between the production and loss of cells. However, the tissues and processes involved in the reactions to disease vary between species and caution over making generalisations between different species was expressed by Ellis, Munro and Roberts (1976).

Lymphoid tissue is associated with organs which have a sinusoidal blood flow, linked via the peripheral circulation enabling lymphoid cells to accumulate in response to antigens (Manning and Turner, 1976). Lymphoid organs contain lymphocytes at various developmental stages and are termed primary or secondary lymphoid organs. Primary lymphoid organs are the major sites of lymphopoiesis, where lymphocytes differentiate from lymphoid stem cells, proliferate and mature into functional effector cells. Secondary lymphoid organs enable the various cells of the immune system to encounter trapped foreign antigen and interact with each other to initiate a co-ordinated immune response aimed at elimination of the pathogen or minimising damage. These functions are performed by phagocytic macrophages, antigen presenting cells and mature T and B lymphocytes.

The thymus, a primary lymphoid organ, appears relatively unchanged throughout vertebrate evolution. It is important in the continuous production of lymphoid cells and may or may not be divided into distinct cortex and medullary regions in fish. From its initially superficial location close to the buccal cavity, the thymus may undergo involution with age or season making it more difficult to locate in older fish (Honma and Tamura, 1984). No antigen processing or phagocytic activity has been reported in the fish thymus (Ellis, 1980).

Secondary lymphoid organs include the spleen and kidney. The spleen, found in all jawed fishes, shows considerable inter-species differences (Haider, 1966). Circulation of blood within this organ is described as open and red pulp consists of a spongy cellular reticulum and large blood sinuses through which erythrocyte rich blood percolates (Yoffey, 1929). It acts as an accessory haemopoietic organ and site of erythrocyte storage, breaking down old erythrocytes and recycling iron (Agius, 1979). Up to a quarter of the total blood cell volume may be held within the spleen and released into the circulation during stress (Fänge and Nilsson, 1985). Splenic antigen binding

cells and antibody producing cells have been seen (Rijkers, Frederix-Walters and van Muiswinkel, 1980) with reports of pyroninophilic cells occurring following antigenic stimulation (Secombes, Manning and Ellis, 1982a). Terminal capillaries (ellipsoids) and melanomacrophage centres (MMCs) are often surrounded by lymphoid tissue (Zapata, 1982).

Along with its excretory function the kidney is an important lympho-myeloid organ, suggested to be analogous to mammalian lymph nodes and bone marrow and functioning as a blood forming organ (Zapata, 1979). It is divided into the predominantly lymphoid anterior kidney (pronephros) and the posterior kidney (mesonephros) which contains lymphoid tissue within the inter-tubular spaces. Many studies have suggested that the kidney is an important antibody producing organ in fish (Manning, 1994). Melanin containing cells are also found within discrete melanomacrophage centres (MMCs).

Turbot lymphoid organs were examined histologically and descriptions of the gross morphology and lymphoid elements of the thymus, spleen and kidney are presented.



## **CHAPTER 4**

### **4.2 MATERIALS AND METHODS**

Sections of the Materials and Methods Chapter of specific relevance to this chapter are:

#### **2.1 FISH**

#### **2.2 HAEMATOLOGICAL STUDIES**

##### **2.2.2 TISSUE SAMPLING**

##### **2.2.2.1 Lymphoid tissue**

##### **2.2.2.2 Tissue imprints**

#### **2.3 LIGHT MICROSCOPY**

#### **2.4 HISTOCHEMICAL STAINING**

## **CHAPTER 4**

### **4.3 RESULTS**

#### **4.3.1 THYMUS**

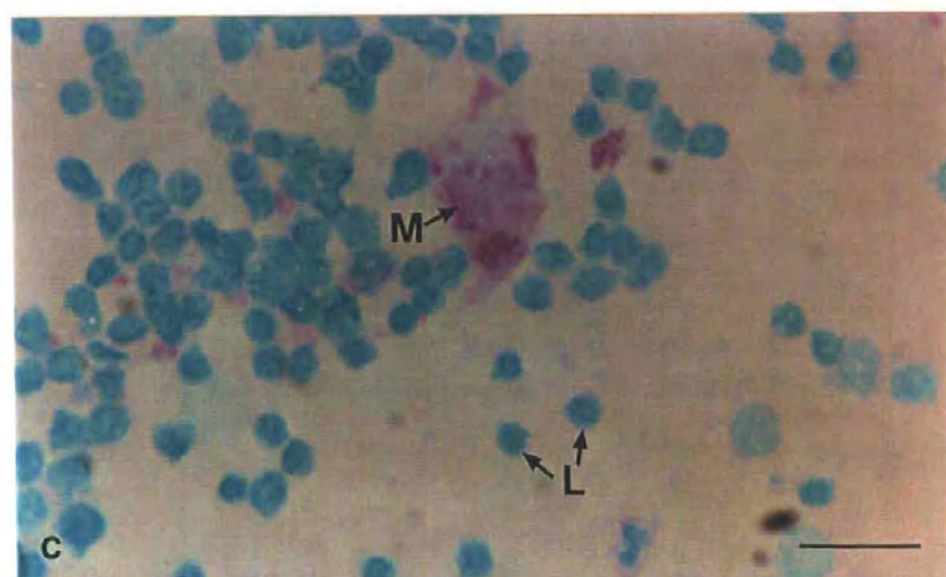
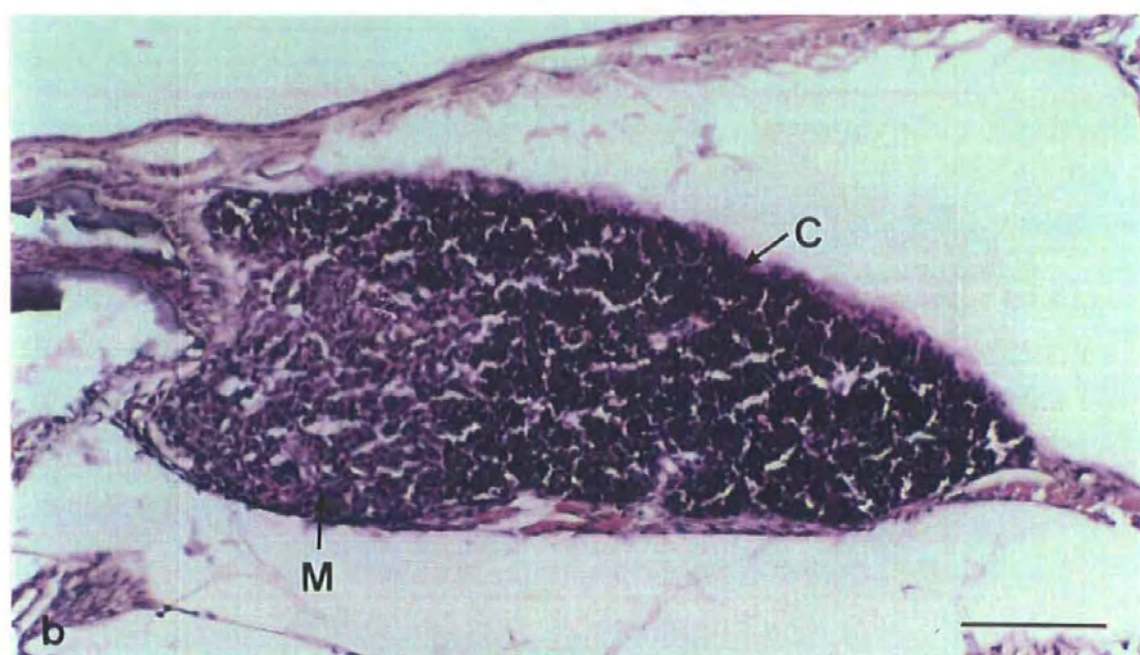
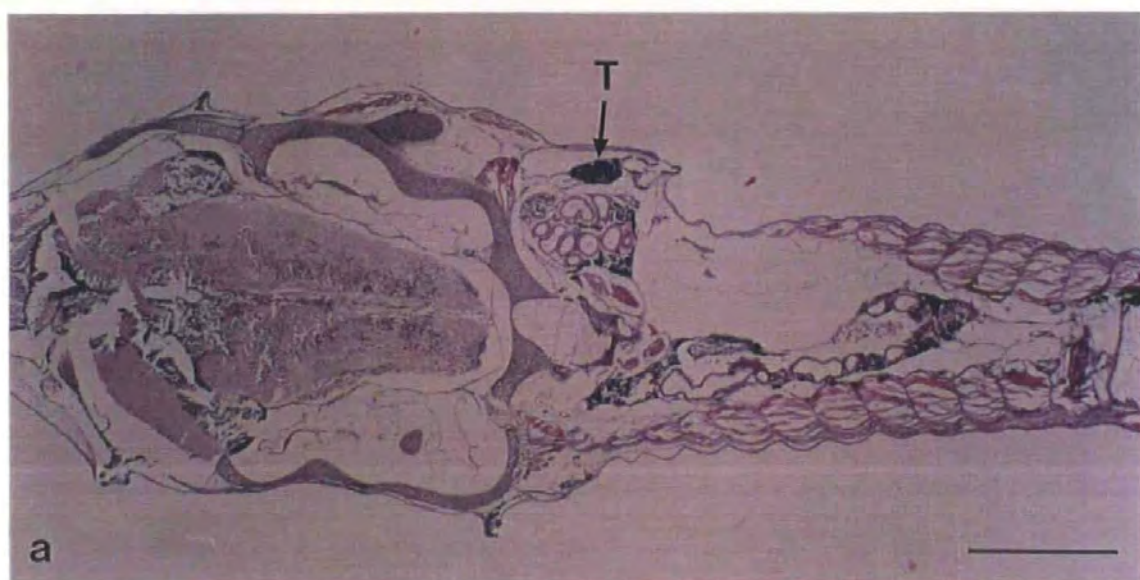
The turbot thymus was identified as a bilaterally paired organ, located at the superior corner of the opercular cavity (Figure 12a), where the operculum joins the cranium. It was small, opaque and pale in colour lying immediately below the epithelium of the branchial cavity. This made the thymus difficult to locate with the naked eye but it could be found histologically.

Fundamentally lymphoid in nature, the thymus was encased within a thin epithelial capsule and supported by a connective tissue network. It was well vascularised and comprised distinct cortex and medullary regions (Figure 12b). The cortex, which was outermost, was densely packed with immature thymocytes with their characteristically dark nuclei and small amounts of pale staining cytoplasm. This region partly surrounded the more median medullary zone, which comprised epithelial cells, small, more mature lymphocytes (7µm) and macrophages, the contents of which stained positively with PAS in tissue imprints (Figure 12c).

Occasionally, large cells with an eosinophilic cytoplasm and large nucleus, resembling mammalian myoid cells, were also observed. No structures resembling the Hassall's corpuscles of mammals were seen.

**Figure 12    Thymus**

- a            Location of the thymus (T). (H&E)**  
**(Bar represents 1mm).**
  
- b            Transverse section through the thymus showing distinct cortex**  
**(C) and medullar (M). (H&E).**  
**(Bar represents 200µm).**
  
- c            Tissue imprint of the thymus comprising small lymphocytes (L),**  
**and PAS positive macrophage (M). (Methyl green / PAS).**  
**(Bar represents 10µm).**



#### 4.3.2 SPLEEN

The turbot spleen, an elongated, flattened organ, was positioned centrally within the peritoneal cavity lying beneath to the liver (ie towards the blind side). It was deep red in colour, containing a large volume of blood and was contained within a thin fibrous connective tissue capsule. Microscopically, blood vessels were clearly seen emanating throughout the structure.

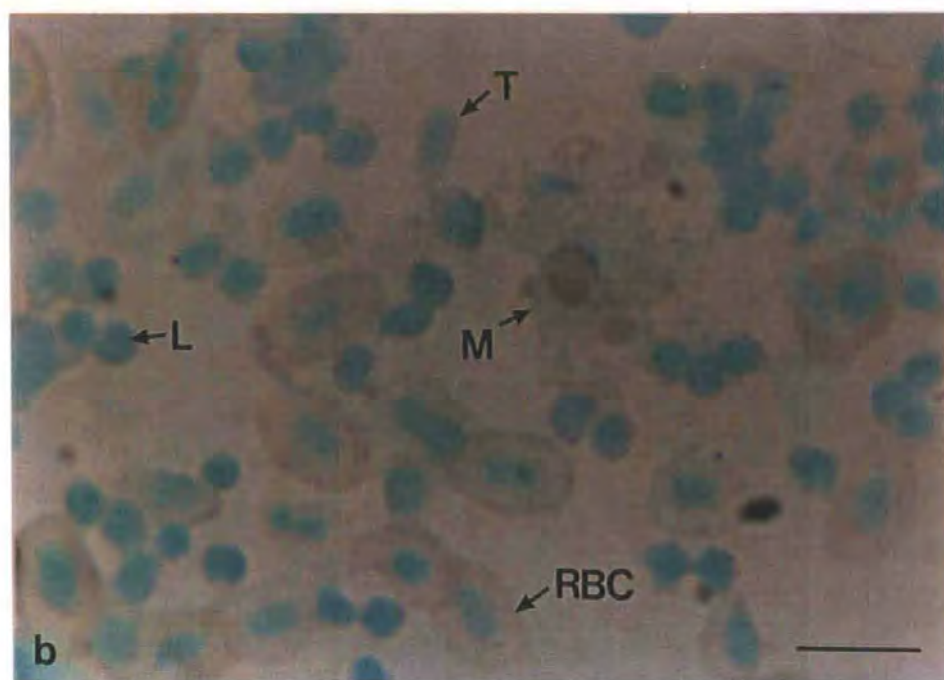
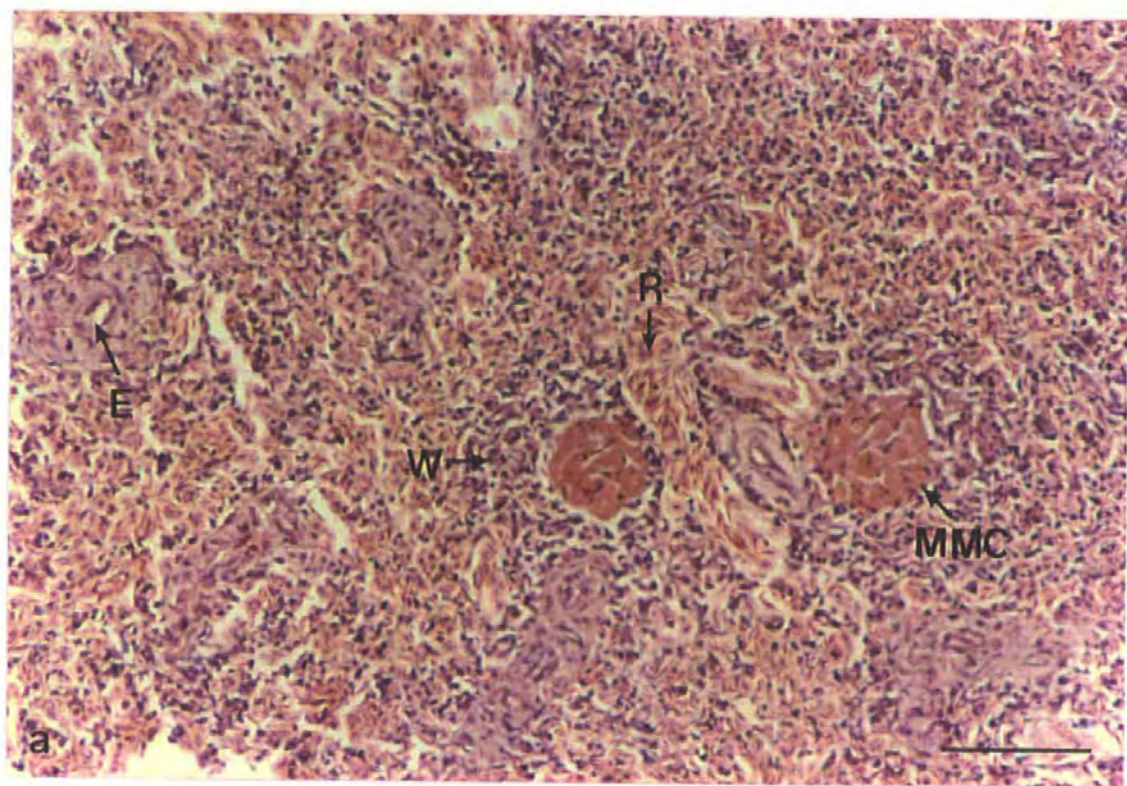
The spleen contained immature red and white cells with some differentiation into diffuse areas of red and white pulp (Figure 13a) which were not macroscopically distinguishable. The red pulp was made up of blood sinuses containing large numbers of erythrocytes (the commonest cellular component of the spleen) and lined by macrophages and granulocytes. Arterioles branched throughout the splenic pulp, the terminal ends of which could be seen as ellipsoids. These were characterised by the presence of a thick wall and narrow lumen. Ellipsoids were surrounded by narrow lymphoid zones of white pulp, reticulin fibres and macrophages. Distinct spherical aggregates of melanin containing cells which in size (20 - 65 $\mu$ m) and structure resembled macrophages, were identified as melanomacrophage centres (MMCs) (Figure 13a). The remainder of the lymphoid elements were scattered in small foci throughout the parenchyma and around the MMCs.

Imprints of the spleen confirmed the identity of cell types present. These included lymphocytes, thrombocytes, macrophages and mature erythrocytes (Figure 13b).

**Figure 13    Spleen**

- a**            Diffuse areas of splenic red (R) and white pulp (W), pigmented melanomacrophages (MMC) and ellipsoids (E). (H&E).  
(Bar represents 200µm).
- b**            Tissue imprint of the spleen comprising mature erythrocytes (RBC), macrophages (M), thrombocytes (T) and lymphocytes (L). (Methyl Green / PAS).  
(Bar represents 10µm).





### 4.3.3 KIDNEY

The kidney occupied a position dorsal to the body cavity beneath the vertebral column and curving along the length of the peritoneal cavity. Anteriorly, the left and right components of the kidney diverged laterally and widened to their broadest point as they curved around the cavity. The anterior areas were not morphologically distinct from the rest of the organ (posterior kidney). The kidney was purple-red in colour and covered by a fibrous renal capsule. Macroscopically, the kidney appeared as a single structure, however it was composed of two, almost fused, sections. Functions of the anterior and posterior regions of the kidney, distinguishable microscopically, were predominantly blood forming and excretory respectively, with no apparent demarcation between these areas.

Anteriorly, the kidney was composed of extensive haemopoietic tissue (Figure 14a) with very few renal tubules and large amounts of connective tissue and blood vessels. The haemopoietic component which formed the parenchyma of the head kidney, occurred between the sinusoids. Narrow blood sinuses formed by cords of reticulo-endothelial cells and leucocytes, notably lymphocytes and granulocytes, radiated from thin walled blood vessels throughout the haemopoietic parenchyma (Figure 14b). Haemopoietic and lymphoid cells observed included melanomacrophages, which were most prominent, and small and large lymphocytes at different stages of development.

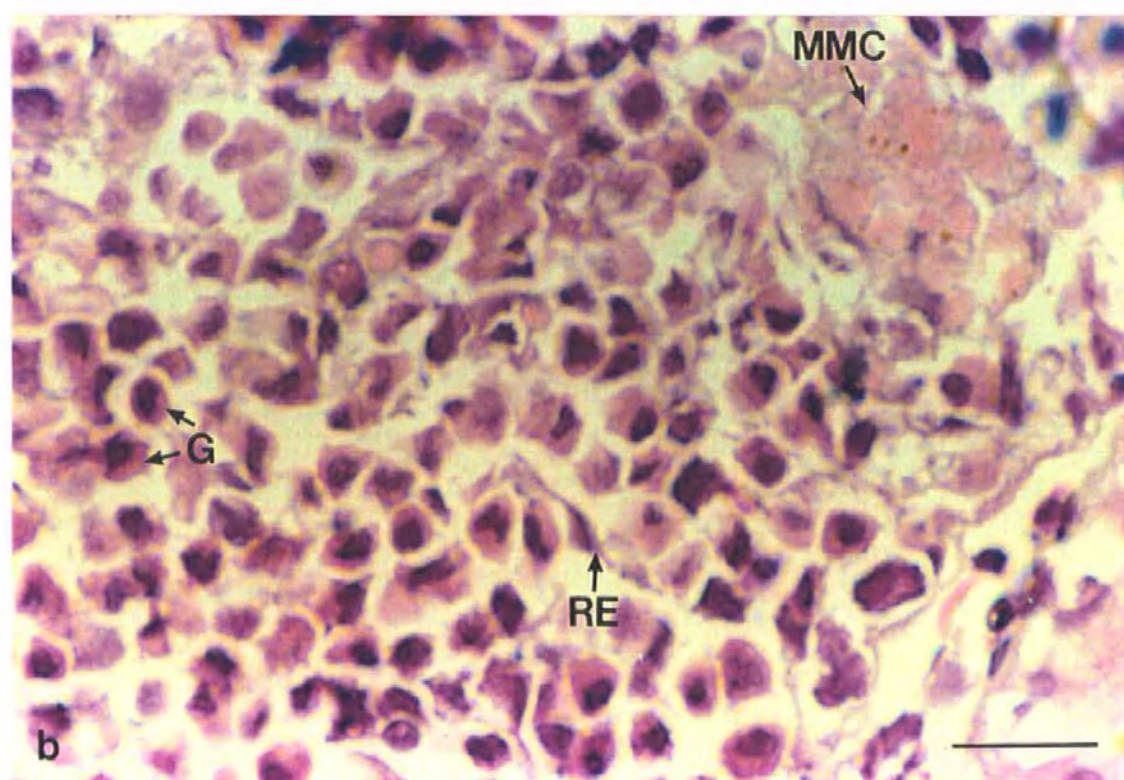
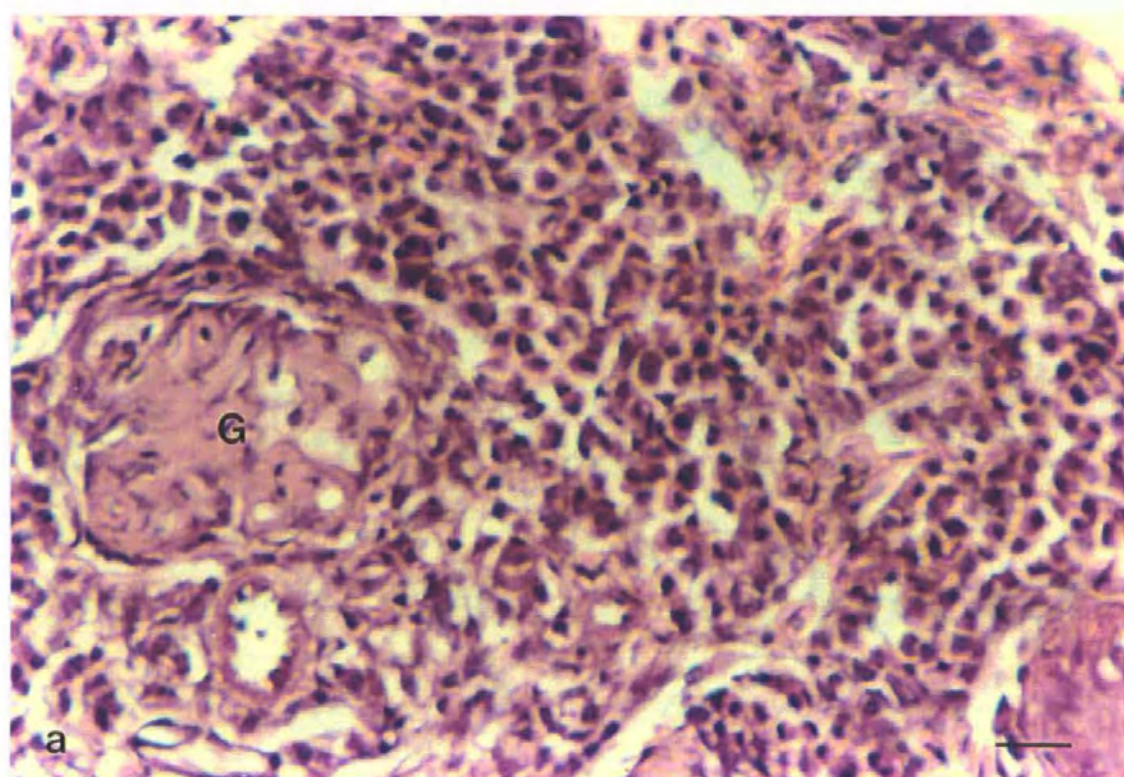


**Figure 14    Anterior Kidney**

**a**            Haemopoietic tissue of the anterior kidney. Glomerulus (G). (H&E).

**b**            Haemopoietic parenchyma containing reticuloendothelial cells (RE), developing granulocytes (G) and melanomacrophage centres (MMC). (H&E).

(Bars represent 50µm)



The posterior kidney was composed of 2 functional units, namely the excretory component which consisted of kidney tubules, Bowman's capsule and glomerulii and the haemopoietic component interspersed between the renal tubules.

In cross-section, profiles of the collecting tubules were prominent because of their large lumina and tall columnar epithelium (Figure 15a). The glomerulii were seen in section as round bodies consisting of capillaries containing red blood cells, bound by thin spaces and encased within Bowman's capsules.

Kidney imprints confirmed the presence of large numbers of developing granulocytes (Figure 15b).

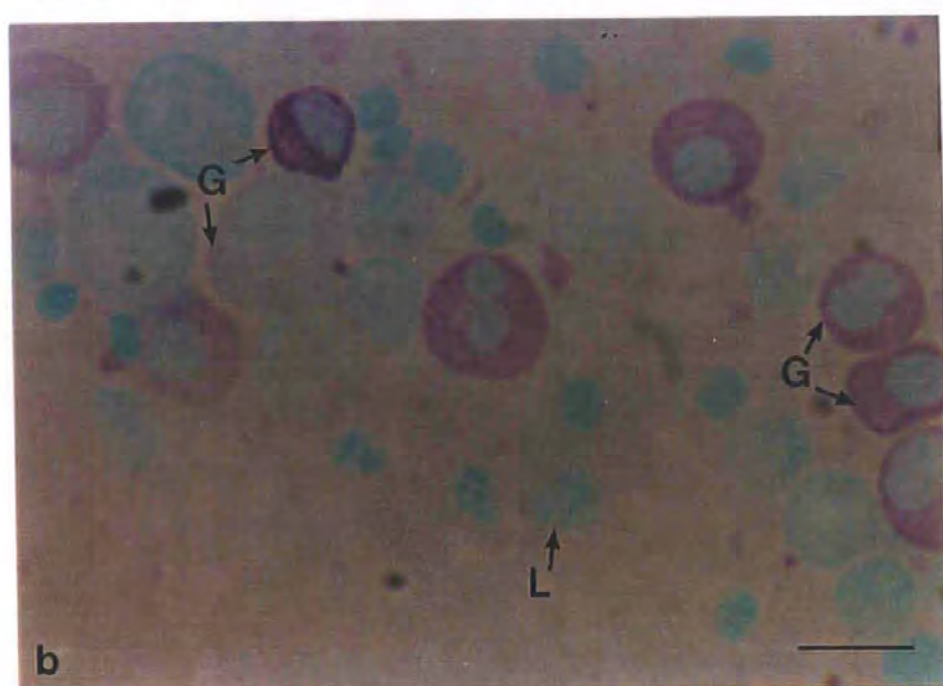
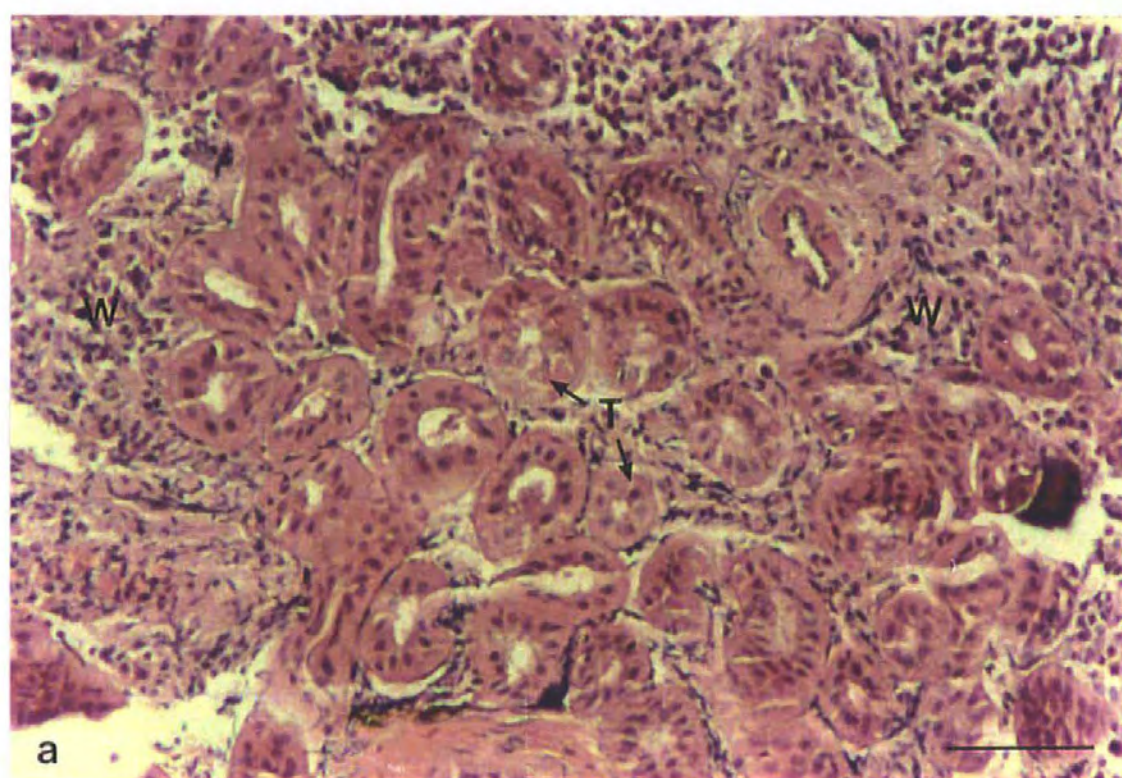
#### **4.3.4 OTHER TISSUES**

In addition, serial sections of other tissues including the liver, heart and gill were examined for aggregations of lymphocytes or lymphoid tissue. None were found.

**Figure 15    Posterior Kidney**

- a            Numerous renal tubules (T) interspersed with lymphoid tissue white pulp (W). (H&E).  
(Bar represents 100μm).**
  
- b            Kidney tissue imprint containing lymphocytes (L) and large numbers of granulocytes (G) at various developmental stages. (Methyl Green / PAS).  
(Bar represents 10μm).**





## CHAPTER 4

### 4.4 DISCUSSION

The principal lymphoid organs of the turbot, a phylogenetically advanced teleost, included the thymus, spleen and kidney. There were distinct similarities with their mammalian equivalents and with the organs of other bony fish, although in many species of fish the details of cellular organisation within lymphoid tissue is species dependent (Rijkers, 1982).

The position of the turbot thymus close to the branchial cavity of the gills is similar to that reported in other fish species (Groman, 1982; Yatsutake and Wales, 1983). Lymphoid cells positioned in the outermost region of the thymus were separated from the external environment by only a thin layer of epithelial cells which constituted the capsule. Such an exposed location of the thymus in the turbot is in contrast to its location in man where it is situated high up in the thorax. However, its positioning is common to many fish species. Indeed, Chilmonczyk (1985) reported the presence of 15µm pores within the epithelial capsule of the thymus of young rainbow trout where tissues appear to lay directly exposed to the external environment. Such exposure of the thymus may play a role in direct immunostimulation of thymocytes in the juvenile animal by water-borne antigens (Yoffey, 1929).

The presence of distinct cortex and medullary zones in the turbot thymus confirms the findings of Sailendri and Muthukkaruppan (1975) in the tilapia, *Sarotherodon mossambicus*, Groman (1982) in the striped bass, *Morone saxatilis*, Bly (1985) in the viviparous blenny, *Zoraces viviparous*, Fänge and Pulsford (1985) in the angler fish, *Lophius lophius*, Fänge (1986) in the sturgeon, *Acipenser transmontanus*, Mughal and Manning (1986) in the grey mullet, *Chelon labrosus* and O'Neill (1989) in the Antarctic teleost, *Harpagifer antarcticus*. However, this structure is not always well defined

in fish (Mughal and Manning, 1986) and a distinction between cortex and medulla could not be made in the Atlantic salmon (Ellis, 1977b) and rainbow trout (Grace and Manning, 1980; Chilmonczyk, 1985). Nevertheless, the differentiation of these tissues in the turbot and in other fish species does not necessarily reflect functional roles similar to those associated with these structures in mammals.

Large cells resembling myoid cells were here observed in the medullary tissue of the turbot thymus. Such cells have been reported to occur in the thymus of other fish species (Zapata, 1980) as well as the mammalian thymus. Their role in mammals remains uncertain although Fänge and Pulsford (1985) speculated that the role of these cells in the angler fish thymus was in the release of lymphocytes into the circulation. Further studies, including electron microscopy, would be necessary before the identity of these cells in the turbot could be confirmed.

The apparent absence of structures resembling Hassall's corpuscles in the turbot thymus is in keeping with the observations in many other teleosts (Zapata, 1981a; O'Neill, 1989; Chilmonczyk, 1985, 1983). However, these have been recorded in other fish species (Hibiya, 1982) including tilapia (Sailendri and Muthukkaruppan, 1975) and in mammals. Their significance remains unclear, although a possible role in mammals for antigen accumulation has been suggested (Kendall, 1981).

Difficulties during the present study in locating the thymus macroscopically in adult turbot may be due to involution of the organ with age, a process characterised by a reduction in size, proliferation of connective tissue and a decrease in thymocyte number as reported in a number of fish species (McCumber, Sigel, Trauger and Cuchens, 1982), including the rainbow trout (Tatner and Manning, 1983).

The structural organisation of the turbot spleen appears similar to that of most teleosts, consisting predominantly of red pulp, blood vessels, ellipsoids and small accumulations of lymphoid tissue (white pulp) (Manning, 1994). In the turbot, the white pulp was primarily found in discrete nodes surrounding ellipsoids and MMC's, similar to many other fish (Zapata, 1982; Ferguson, 1976; Sailendri and Muthukkaruppan, 1975). In other fish, however, the white pulp is more diffuse (Lamers, 1985; Ellis *et al*, 1976). Considerable species variation in the structure and function of the spleen has been reported amongst teleosts (Fänge and Nilsson, 1985). Increasing organisation has been reported in advanced teleosts, nevertheless, the structure of the spleen is generally less organised than in mammals (Ellis, 1978).

As in the rainbow trout (Tatner and Manning, 1982), the capsule of the turbot spleen was shown here to consist of a thin epithelial covering. This was not as thick or prominent as the capsule of the mammalian spleen (Yatsutake and Wales, 1983) and no muscular tissue was seen.

Ellipsoids are conspicuous within the turbot spleen as is common in many fish including the plaice (Ellis *et al*, 1976) but are less obvious or even absent in some species (Yoffey, 1929). Ellipsoids have been suggested to play a role in antigen trapping with subsequent transport of the material by macrophages to the melanomacrophage centres (Ferguson, 1976), a view supported by Secombes, Manning and Ellis (1982b) and van Muiswinkel *et al* (1991).

The spleen is the major site of destruction of aged erythrocytes and other effete cells in teleosts (Fänge and Nilsson, 1985). It is seen as the largest filter of the blood responsible for the removal of foreign substances and damaged or old erythrocytes from the circulation. Since the diameter of erythrocytes is larger than that of the ellipsoids through which it must pass compression occurs. Any fragments or fragile cells are then removed from



the system and the presence of disintegrating erythrocytes within ellipsoid walls has been reported by many.

Phagocytic cells with the capacity to engulf material and effete blood cells from the circulation abound. MMC's were present and well developed in the turbot spleen, being larger than those observed in the pronephros. These findings support those in the plaice (Ferguson, 1976) and striped bass (Groman, 1982). This may reflect a more active role of splenic MMC's in breakdown of effete erythrocytes (Fänge and Nilsson, 1985), which may also account for their higher iron content (Manning, 1994). Many authors have suggested that MMC's play a role in the immune system of fish. Their appearance in the turbot spleen, surrounded by lymphoid cells is indeed similar to that of germinal centres of birds and mammals and would support the suggestion that they are primitive analogues of these structures (Agius, 1980) involved in the production of memory cells (Secombes *et al*, 1982; Lamers, 1985).

The presence of immature erythrocytes and granulocytes in the turbot spleen suggest that this organ may have an erythropoietic and granulopoietic function. This supports the findings reported in various teleosts by Haider (1966). Thrombocytes are also seen within the turbot spleen. Since they are relatively scarce within the other haemopoietic organ, the kidney, the spleen would appear a likely production site as reported in the dogfish (Pulsford *et al*, 1982). In mammals platelets are produced in the spleen where up to one third lie in reserve.

The turbot kidney was shown here to comprise of an anterior lymphomyeloid region (pronephros) and a posterior region which was predominantly excretory in function containing a limited quantity of inter-tubular lymphoid tissue (mesonephros). This is in keeping with the patterns seen in other fish species (Ellis *et al*, 1976; Zapata, 1979). However, the anterior and posterior

regions of the kidney were not discernible macroscopically, in contrast to the findings in the carp and the goldfish, *Carassius auratus* (Hibiya, 1982). Embryologically the anterior kidney has an excretory function (Manning, 1994), however, this is virtually lost in the adult fish, where mainly haemopoietic and lymphoid cells with few or no tubules are seen.

The lymphoid tissue of the turbot pronephros was diffusely distributed, as in the plaice (Ellis *et al*, 1976), the rainbow trout (Grace and Manning, 1980) and the roach, *Rutilus rutilus*, (Zapata, 1981b). In contrast, in the tilapia (Sailendri and Muthukkaruppan, 1975) and the carp (Lamers, 1985), the lymphoid tissue of this organ was discretely arranged surrounding the MMC's and renal blood vessels. The haematopoietic tissue of the turbot kidney, in keeping with other teleosts (Zapata, 1979; Rijkers *et al*, 1980; Ellis, 1988), resembled the bone marrow of higher vertebrates.

## CHAPTER 5

### ONTOGENIC DEVELOPMENT OF THE LYMPHOID TISSUE

#### 5.1 INTRODUCTION

Turbot, like other flatfish undergo metamorphosis from a symmetrical larva to an asymmetrical form as a juvenile and adult. Metamorphosis occurs as a result of right side migration, where the right eye migrates to lie next to the left on the left side of the body (Wheeler, 1978). The rate at which these and other morphological changes occur during larval development remains under the influence of genetic and perhaps more importantly, environmental factors including temperature, water quality and diet (Jones, 1972, 1973).

The stage of development of internal organs, including lymphoid tissues, is not necessarily linked to age *per se* or to the length and weight of the animal which are also dependent upon environmental factors (Ryland and Nichols, 1967) and time consuming to measure (Ryland, 1966). Thus, if the development of the lymphoid tissues at any time is to be estimated without the need for histological examination, it must be linked to features which are similarly influenced by environmental parameters.

A system of staging larvae has been described for plaice based upon easily externally recognisable morphological features (Ryland, 1966). Al-Maghazachi and Gibson (1984) have characterised the metamorphosis of larval turbot into 5 developmental stages using a similar system. The staging / numeration system they proposed was adopted here and appears in parentheses. This provided a visual system based upon external features of the juvenile fish to facilitate correlation between externally recognisable morphological characteristics and development of the lymphoid tissues.

Accurate determination of the stage of development of lymphoid organs can be of the utmost importance in the fish farming environment. Timing of vaccination of fish against various known pathogens is critical if the problem of immunotolerance caused by premature vaccination are to be avoided (Ellis, 1988b).

Several studies have been conducted describing the ontological development of the lymphoid tissues of fish. Some of the descriptions which have been made include *Brachydaio rerio* (Al-Adhari and Kunz, 1977), salmon (Ellis, 1977), rainbow trout (Grace and Manning, 1980), carp (Botham and Manning, 1981), *Nothobranchius gaentheri* (Cooper; Zapata; Garcia Barrutia and Ramirez, 1983), viviparous blenny (Bly, 1985), *Harpagifer antarcticus* (O'Neill, 1989), sea bream (Jósefsson and Tatner, 1993), dogfish (Lloyd-Evans, 1993) and the flounder, *Platichthys flesus* (Pulsford, Thomlinson, Lemaire-Gony and Glynn, 1994).

In this study length, weight and gross morphology were assessed at regular intervals from 24h post-hatch to Day 63 (the completion of metamorphosis). Histological samples were taken at corresponding time intervals to enable development of the lymphoid tissues to be assessed and correlated with gross morphology.

## **CHAPTER 5**

### **5.2 MATERIALS & METHODS**

Sections of the Materials and Methods Chapter of specific relevance to this chapter are:

#### **2.1 FISH**

#### **2.2 HAEMATOLOGICAL STUDIES**

##### **2.2.2 TISSUE SAMPLING**

##### **2.2.2.1 Lymphoid tissue**

#### **2.3 LIGHT MICROSCOPY**

##### **2.3.1 ONTOGENIC STUDIES**

## **CHAPTER 5**

### **5.3 RESULTS**

#### **5.3.1 MORPHOLOGICAL DEVELOPMENT**

Development followed the basic pattern described by Al-Maghazachi and Gibson (1984) and outlined in Table 3. At hatching larvae were symmetrical with an eye on either side of the head and a ventrally positioned mouth. By the end of Stage 1 (24h post-hatch to Day 3) the yolk sac was lost and both mouth and anus were open with the onset of first feeding. Stage 2 (Day 4 to Day 10) was characterised by development of the intestine and appearance of the swim bladder. Fin rays were first seen in the shortest stage, Stage 3 (Day 11 - Day 12). Metamorphosis which had a profound effect on the symmetry of the skull and whole fish had begun by Stage 4 (Day 13 - Day 23) when right eye migration commenced and the notochord had bent upwards. Continued migration of the right eye, loss of pigmentation from the lower surface, widening and flattening of the body and development of the pelvic fins indicated completion of metamorphosis by the end of Stage 5 (Day 28 - 63). Age and length are correlated with the five developmental stages in Table 4 which are shown in Figure 16.

#### **5.3.2 HISTOLOGY**

##### **5.3.2.1 Thymus**

At the first sampling interval of 24h (Stage 1a) post-hatch, the thymus appeared as a slight thickening of the epithelium lining the dorso-anterior

Table 3

DEVELOPMENTAL STAGES OF LARVAL TURBOT, BASED UPON MORPHOLOGICAL FEATURES (AFTER AL-MAGHAZACHI AND GIBSON, 1984)

Stage	Sub-stage	Morphological characteristics
1		<b>Larvae symmetrical, yolk sac present</b>
	1a	Head bent down and attached to yolk sac; large quantities of yolk, 1 oil globule present; gut almost straight tube.
	1b	Head forward pointing, free from yolk sac; gut thickening in middle, curved posteriorly downwards; anus not reaching edge of primordial marginal fin.
	1c	Gut thickening in centre slightly bent ventrally rectum distinct; anus reaching marginal fin; mouth slightly open; primordial dorsal fin extends above eyes; larvae not feeding.
	1d	Yolk sac very small; ventral wall of abdominal cavity almost straight; intestine fully developed and bent ventrally, mouth and anus open; active feeding; if food abundant yolk sac with oil globule may persist into next stage.
2		<b>Larvae symmetrical, spine and swim bladder development</b>
	2a	Yolk sac completely absorbed or small sac around oil globule; gut almost reaching abdominal wall; few spines apparent on operculum.
	2b	Gut looped; intestine reaches abdominal wall; small swim bladder seen above stomach in gastrointestinal space; numerous spines on operculum; bony ridged line above eyes.
	2c	Swim bladder larger, 2 rows of spines on operculum 2-3 spines on inter-operculum; primordial dorsal fin extends above auditory sac.
3		<b>Appearance of fin rays, notochord straight</b>
	3a	Swim bladder fully inflated; hypural fin rudiment very small, first 1-2 fin rays barely visible; margin of fin not extended.
	3b	4-7 fin rays present; margin of fin slightly extended.
4		<b>Asymmetry and eye migration, notochord slanted dorsally</b>
	4a	Notochord caudally bent upwards by $<45^\circ$ ; numerous fin rays present; margin of fin fold with further extension; caudal fin exhibiting asymmetrical features.
	4b	Notochord sloped upward $45^\circ$ or more but $<90^\circ$ ; rapid development of hypural fin; eye migration commences (right eye in higher position than left).
	4c	Right eye positioned further upwards but not visible from left side; 3-4 spines on underside of each jaw.
	4d	Notochord bent straight upwards; caudal fin rays fully developed; few fin rays visible in ventral fin; upper edge of right eye can be seen from left side; 5-6 spines on each jaw.
5		<b>Eye migration complete, spines and swim bladder resorbed</b>
	5a	Half of right eye visible from left side; dentary spines completely resorbed; resorption of other spines commenced.
	5b	Right eye on top of head (now termed upper eye); spines smaller and fewer.
	5c	Right eye situated entirely on left side but still near upper edge, all remaining spines fully resorbed.
	5d	Upper eye placed away from upper edge; swim bladder disappeared; dorsal fin extends to above front of eyes; metamorphosis now complete.

Table 4

## AGE, LENGTH AND DEVELOPMENTAL STAGES IN JUVENILE TURBOT

STAGE	SUBSTAGE	AGE (post-hatch)	LENGTH * mm ( $\pm$ SD)
1			
	1a	24h	2.6 ( $\pm$ 0.1)
	1b	Day 2	2.8 ( $\pm$ 0.3)
	1c	Day 2 - 3	3.1 ( $\pm$ 0.2)
	1d	Day 3	3.2 ( $\pm$ 0.4)
2			
	2a	Day 4	3.7 ( $\pm$ 0.2)
	2b	Day 8	4.5 ( $\pm$ 0.7)
	2c	Day 10	4.9 ( $\pm$ 0.5)
3			
	3a	Day 11	5.2 ( $\pm$ 0.4)
	3b	Day 12	5.8 ( $\pm$ 1.0)
4			
	4a	Day 13	7.3 ( $\pm$ 0.6)
	4b	Day 18	8.8 ( $\pm$ 1.1)
	4b	Day 20	11.8 ( $\pm$ 1.2)
	4d	Day 23	13.0 ( $\pm$ 1.0)
5			
	5a	Day 28	14.1 ( $\pm$ 1.4)
	5b	Day 35	19.0 ( $\pm$ 1.7)
	5c	Day 49	26.5 ( $\pm$ 0.6)
	5d	Day 63	41.0 ( $\pm$ 2.6)

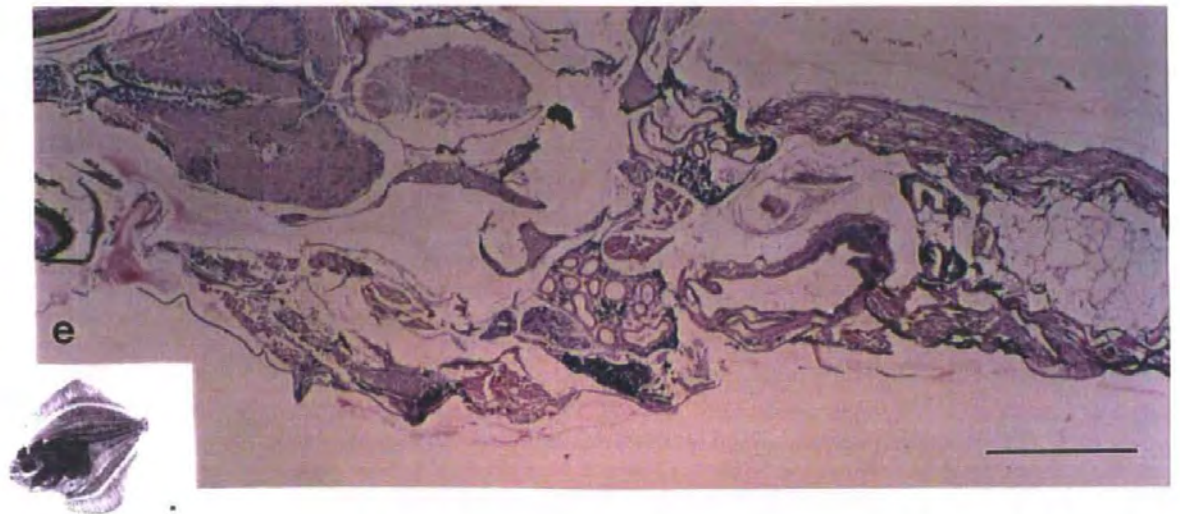
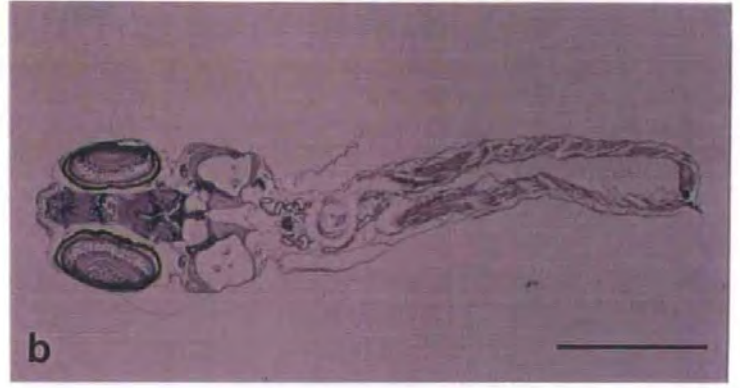
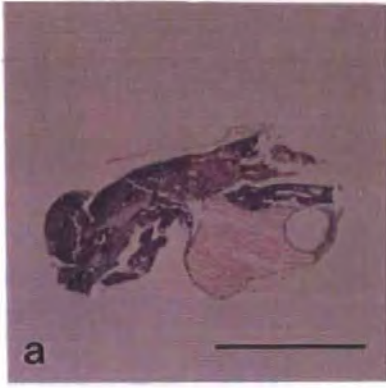
\* Sample size (n) = 3 - 15 fish



Figure 16 Developmental stages of juvenile turbot.

- a Stage 1. Larvae symmetrical, yolk sac present. (H&E).
- b Stage 2. Larvae symmetrical, spine and swim bladder forming. (H&E).
- c Stage 3. Appearance of fin rays, notochord straight. (H&E).  
Inset: shows gross morphology.
- d Stage 4. Asymmetry and eye migration, notochord slanted dorsally. (H&E).  
Inset; shows gross morphology.
- e Stage 5. Eye migration complete, spines and swim bladder resorbed. (H&E).  
Inset: shows gross morphology.

(Bars represent 1mm).



section of the pharynx. The cells it contained at this stage were epithelial in appearance. By Day 4 (Stage 2a) the organ had increased in size and some cells resembling those of the lymphoid series observed, although many still resembled epithelial cells (Figure 17a). Between Days 8 and 12 post-hatch (Stages 2b to 3b) a connective tissue capsule formed around the thymus and connective tissue gradually began to infiltrate the parenchyma (Figure 17b). By Day 14 (Stage 4a) all thymic cells were lymphoid in appearance (Figure 17c). The size of the thymus and number of cells it contained continued to increase gradually reaching a maximum at Day 28 (Stage 5a). During Days 28-31 (Stages 5a to 5b), differentiation of the tissue into distinct cortex and medullary regions was evident (Figure 17d). The position of the thymus remained unchanged throughout the study, being separated from the pharyngeal cavity by a thin epithelial layer.

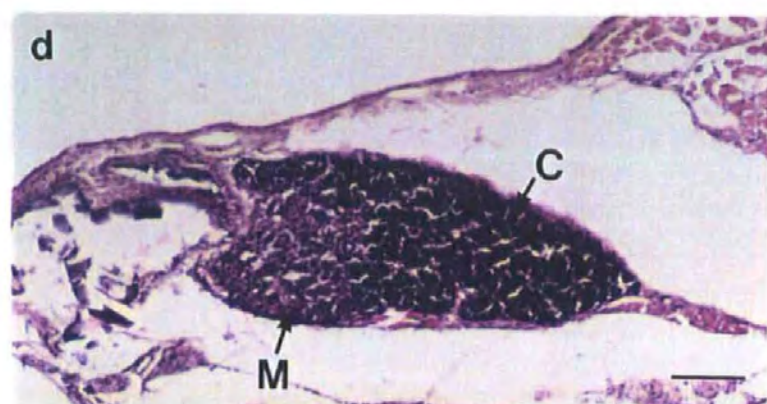
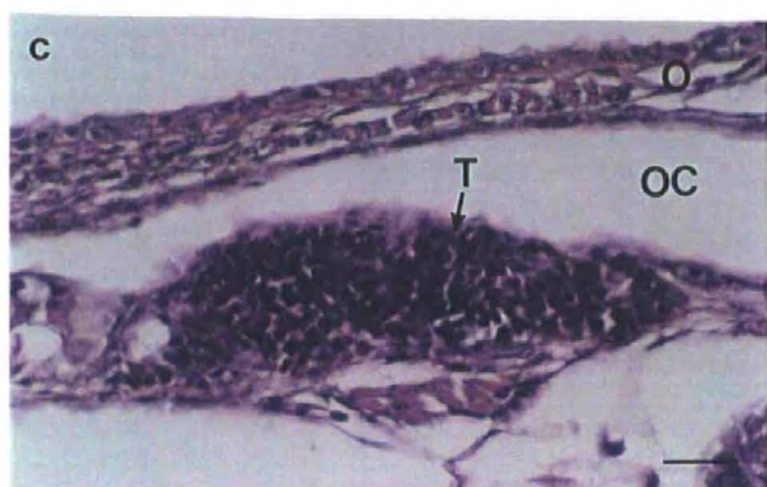
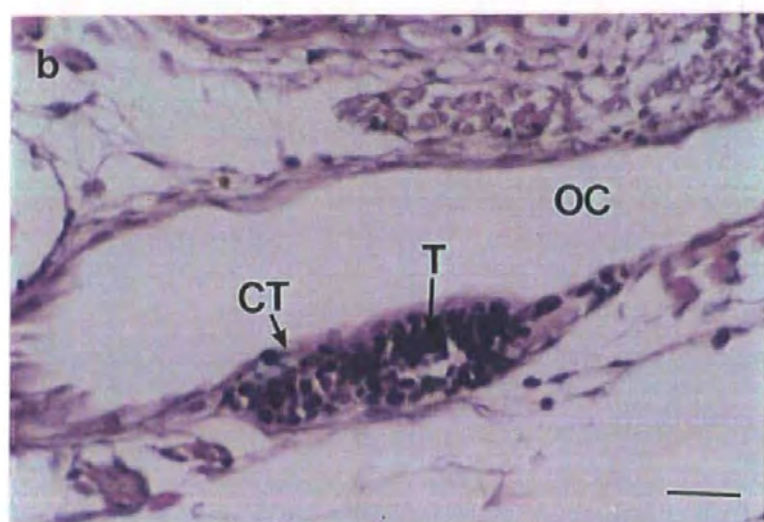
#### **5.3.2.2 Kidney**

The kidney was already well developed by the first sampling interval at 24h post-hatch (Stage 1a). It occupied the length of the peritoneal cavity, running along its dorsal surface. Nephric tubules were present but only very few immature lymphoid cells were seen at this time (Figure 18a). However, large undifferentiated eosinophilic cells were observed, thought to resemble haemopoietic stem cells. By Day 4 (Stage 2a) erythrocytes and lymphoid cells resembling small lymphocytes were observed in the inter-tubule spaces (Figure 18b). The bilateral pronephric region could be recognised at this stage, containing a lower proportion of tubules. From this time forward there was little change in the appearance of this organ except that it gradually increased in size, becoming more densely packed with leucocytes and containing an increased number of nephric tubules (Figure 18c).

**Figure 17    Thymic development.**

- a**            Day 4 (Stage 2a). Thymus (T) has developed from a thickening of the pharyngeal epithelium (E). Opercular cavity (OC), cartilage (C). (H&E). (Bar represents 400µm).
- b**            Day 12 (Stage 3b). Connective tissue capsule (CT) has formed around the thymus (T). Opercular cavity (OC). (H&E). (Bar represents 400µm).
- c**            Day 14 (Stage 4a). Cells of the thymus (T) more lymphoid in appearance. Operculum (O), opercular cavity (OC). (H&E). (Bar represents 400µm).
- d**            Day 31 (Stage 5a/b). Distinct cortex (C) and medullary (M) regions evident. (H&E). (Bar represents 100µm).

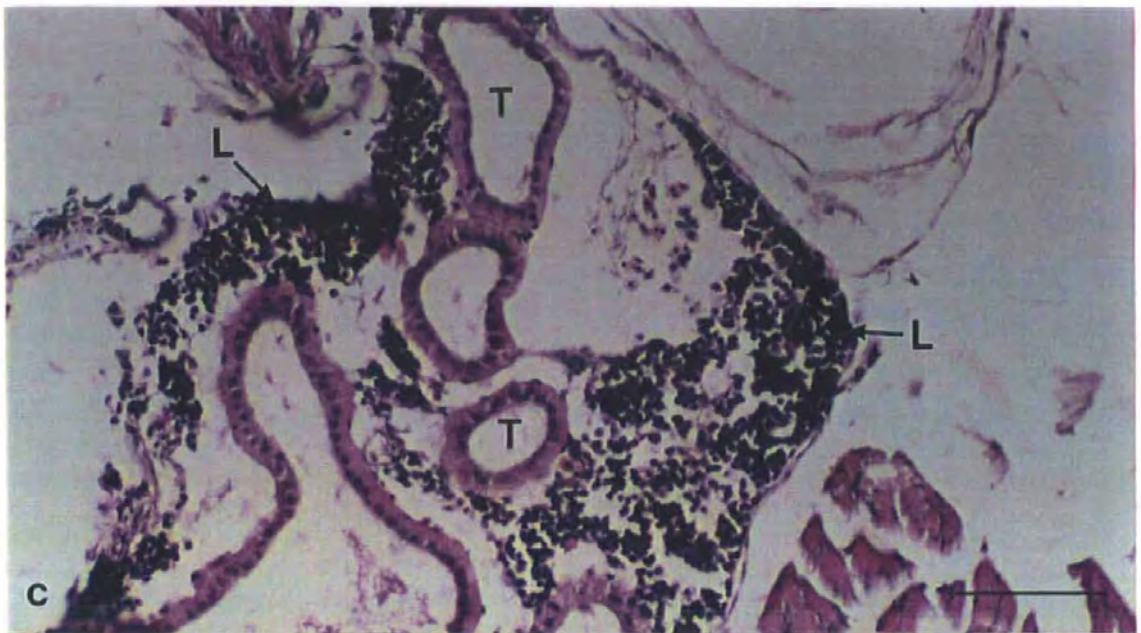
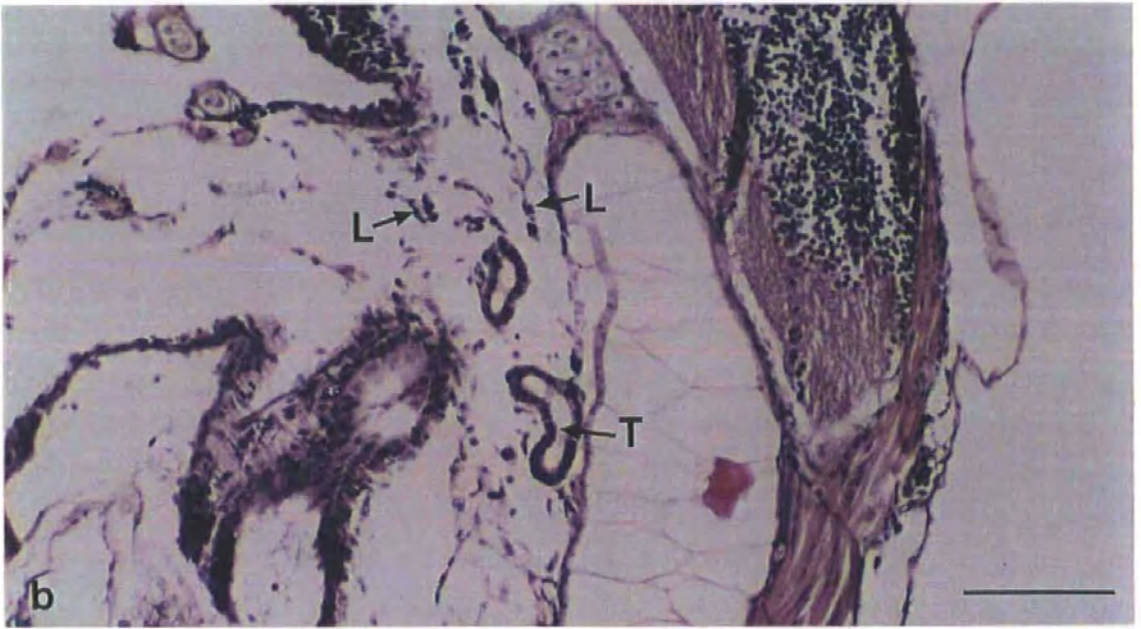
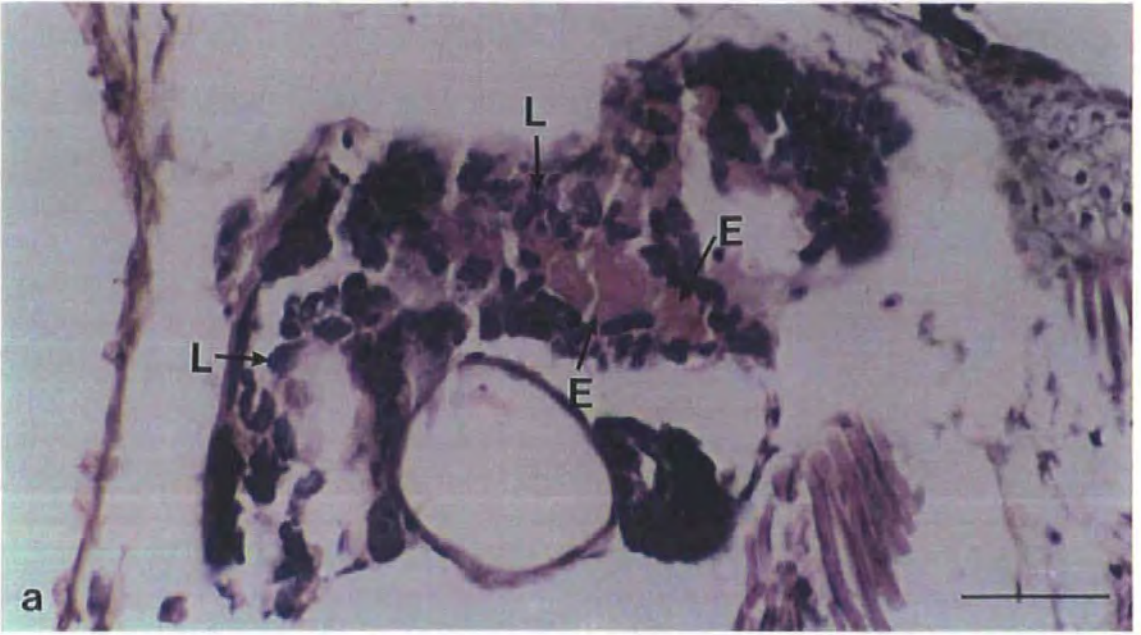




**Figure 18    Kidney development**

- a**            24h post-hatch (Stage 1a). Haemopoietic regions of the kidney comprise immature lymphoid cells (L) and undifferentiated eosinophilic cells (E). (H&E). (Bar represents 800µm).
- b**            Day 4 (Stage 2a). Sparse distribution of lymphoid cells (L) observed between nephric tubules (T). (H&E).  
(Bar represents 400µm).
- c**            Day 28 (Stage 5a). Increased number of nephric tubules (T). Areas of lymphoid tissue (L) now larger and more densely packed. (H&E). (Bar represents 400µm).





### 5.3.2.3 Spleen

At 24h post-hatch (Stage 1a), the splenic rudiment comprised a grouping of large cells, similar in appearance to epithelial cells, which was adjacent to the pancreatic rudiment, in a position anterior to the gut (Figure 19a). This grouping of cells had grown larger by Day 4 (Stage 2a) and consisted of epithelial-like cells and many erythrocytes within large vascular spaces (Figure 19b). The rudiment had formed a definite shape by Day 8 (Stage 2b) and between Day 8 and Day 14 (Stages 2b to 4a) a thin capsule had formed around the spleen (Figure 20a). The organ increased in size up to Day 14 (Stage 4a) when many cells were still epithelial in appearance and a large number of erythrocytes were observed.

By Day 18 (Stage 4b) the cells of the spleen began to take on a more lymphoid appearance although many epithelial-like cells were still present and erythrocytes prominent (Figure 20b). Ellipsoids and the lymphoid component became more obvious by Day 20 (Stage 4c) when the tissue was still surrounded by pancreatic tissue. Tissue of the spleen was well developed by Day 24 (Stage 4d) when distinct red and white pulp and a fibrous structure were evident. The first melanomacrophage centre was observed containing darkly pigmented cells at Day 31 (Stage 5b) (Figure 20c).

Structural development of the thymus, spleen and kidney appeared to be complete by the end of metamorphosis (Stage 5d), when each had taken on the appearance of lymphoid organs seen in adult turbot.

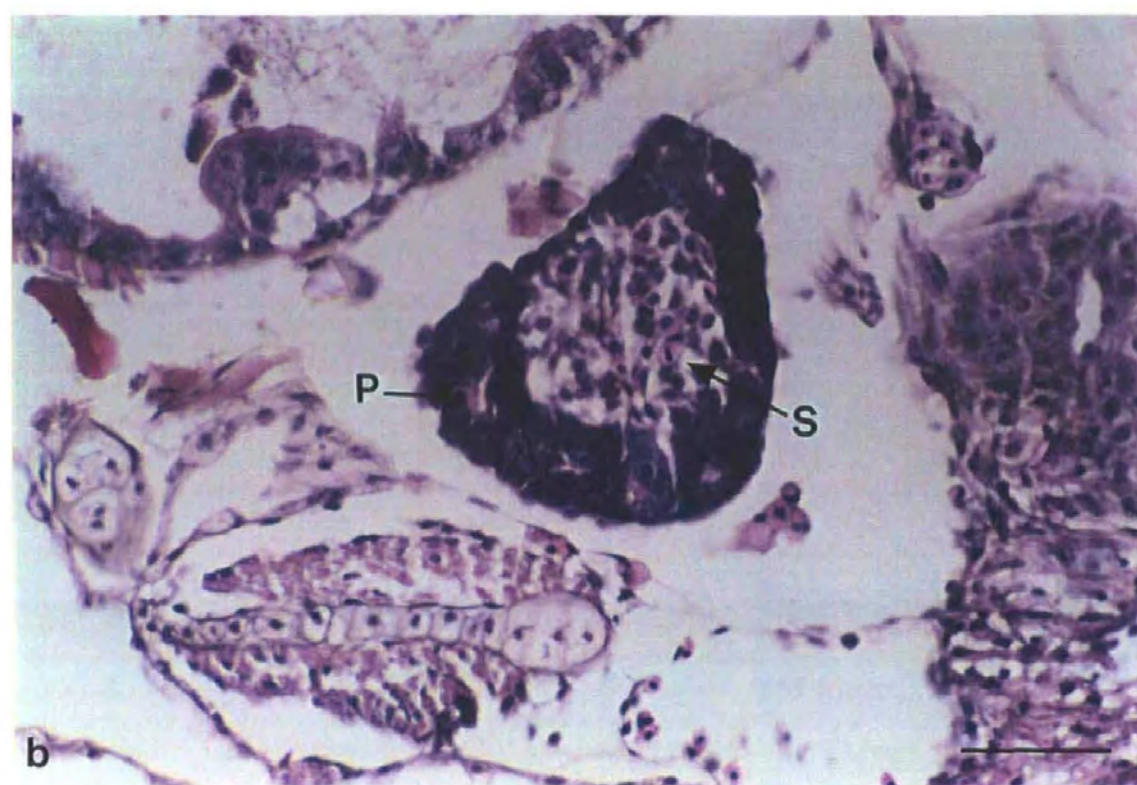
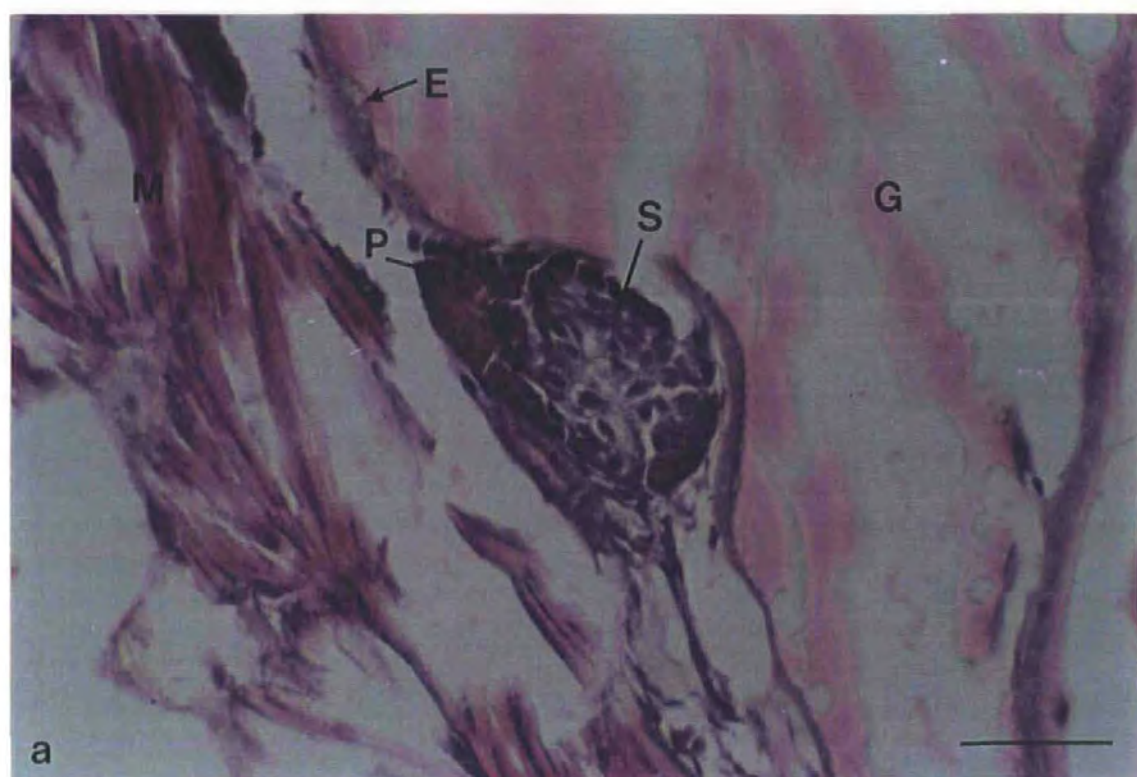
A summary of lymphoid tissue development in larval turbot at the various stages of morphological development is given in Table 5.



**Figure 19     Splenic development I.**

- a            24h post-hatch (Stage 1a).   Splenic rudiment (S), comprising epithelial-like cells. Pancreatic acinar cells (P), epithelial lining of the gut (E), gut lumen (G), musculature (M). (H&E).**
  
- b            Day 4 (Stage 2a).   Splenic rudiment (S) has increased in size and is well vascularised. Pancreatic tissue (P). (H&E).**

**(Bars represent 800µm)**



**Figure 20 Splenic development II.**

- a** Day 12 (Stage 3b). Spleen (S) is now elongate and enveloped in a thin capsule (C). Pancreatic tissue (P), gut (G), liver (L). (H&E). (Bar represents 800µm).
- b** Day 18 (Stage 4b). Spleen (S) now more lymphoid in appearance and well vascularised. Pancreatic tissue (P), gut (G), liver (L). (H&E). (Bar represents 800µm).
- c** Day 31 (Stage 5b). Distinct fibrous structure with red and white pulp evident in the spleen (S). Melanomacrophage centre (MMC), gut (G), liver (L). (H&E). (Bar represents 400µm).



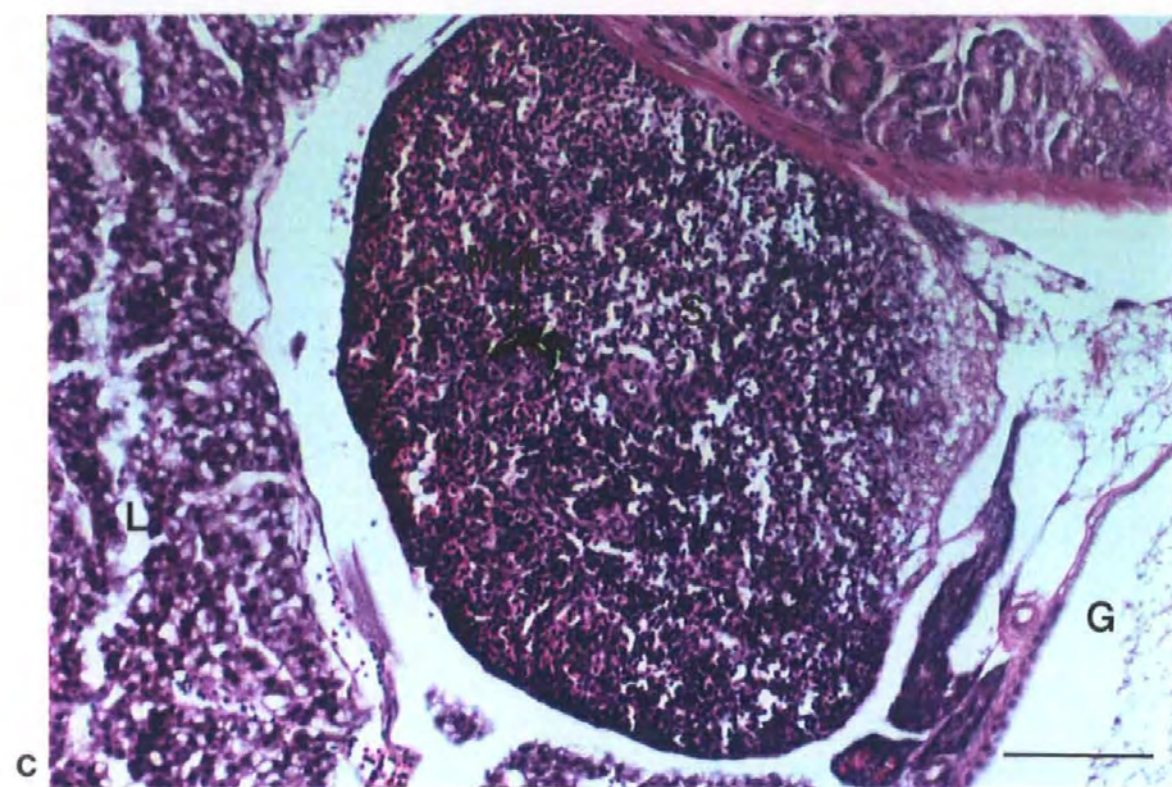
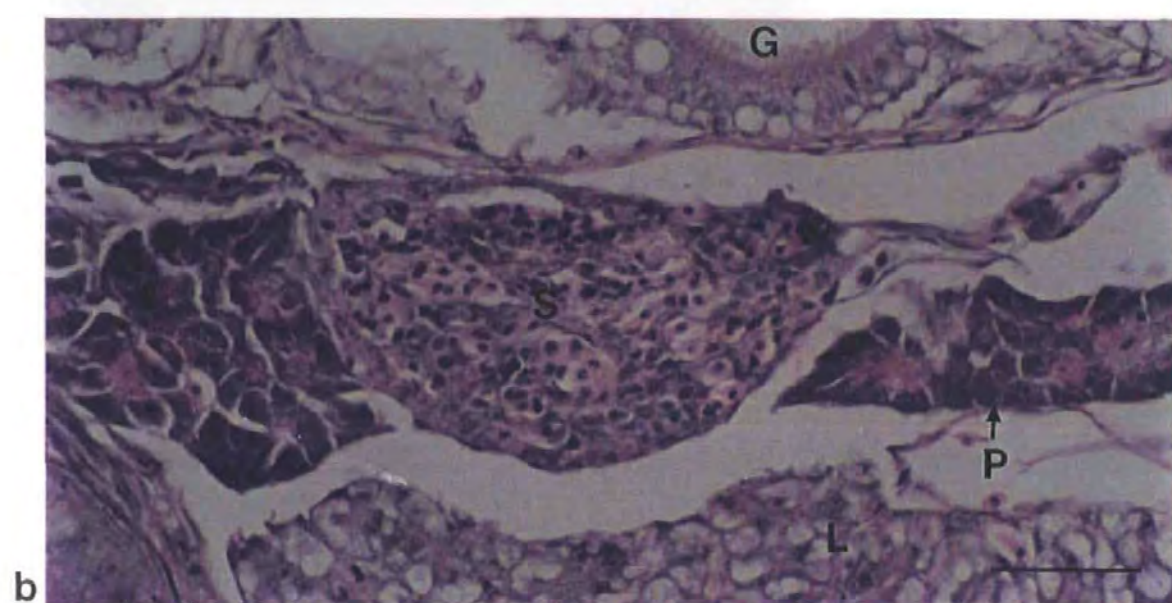


Table 5

CORRELATION BETWEEN MORPHOLOGICAL FEATURES AND  
SUMMARY OF DEVELOPMENT OF LYMPHOID TISSUE IN  
JUVENILE TURBOT.

	<b>MORPHOLOGICAL FEATURES</b>	<b>LYMPHOID TISSUE DEVELOPMENT</b>
<b>STAGE 1</b>	Larvae symmetrical, yolk sac present	Thymic, splenic and kidney rudiments all present
<b>STAGE 2</b>	Larvae symmetrical, spine and swim- bladder development	First lymphoid cells appear in thymus and kidney
<b>STAGE 3</b>	Appearance of fin rays, notochord straight	Kidney increasing in size and lymphoid component
<b>STAGE 4</b>	Asymmetry and eye migration, notochord slanted dorsally	All thymic cells lymphoid, lymphoid cells in spleen develop into white pulp towards end of stage
<b>STAGE 5</b>	Eye migration complete, spines and swimbladder resorbed	Differentiation of thymus into cortex and medulla, first melanomacrophage centre seen in spleen

## CHAPTER 5

### 5.4 DISCUSSION

The turbot thymus appeared to begin its embryological development as a thickening of the pharyngeal epithelium above the gill arches, as is the case in most teleosts (Manning, 1994). These epithelial cells were gradually replaced with those of the lymphoid series with the eventual establishment of a connective tissue framework and capsule. Similar findings have been reported in several other fish species including the salmon (Ellis, 1977), the rainbow trout (Grace and Manning, 1980) and the carp (Botham and Manning, 1981). Unlike other vertebrates the thymus retains its connection with the pharynx, never becoming internalised, instead remaining in a superficial location within the pharyngeal lumen. The turbot thymus continued to increase in size with eventual differentiation of the tissue into cortex and medulla as seen in the adult (described in Chapter 4).

The kidney of the turbot initially comprised nephric tubules and undifferentiated cells, prior to the appearance of lymphoid cells in the inter-tubule spaces. Both the anterior and posterior kidney contained excretory tubules, although the proportion of these in the former diminished with time. This pattern of development has been reported in several other fish species including *Harpagifer antarcticus* (O'Neill, 1989), the carp (Botham and Manning, 1981), the rainbow trout (Grace and Manning, 1980) and the salmon (Ellis, 1977).

The turbot spleen first appeared as a grouping of cells surrounded by the pancreatic rudiment. This is in keeping with the findings in many different animal groups including fish, whereby the splenic rudiment first appears as a mesenchymal condensation close to the pancreatic rudiment (Pitchappan, 1980). Splenic development in the turbot was similar to that of other fish

(Ellis, 1977; Botham and Manning, 1981; O'Neill, 1989) in that it assumed a definite shape, became encapsulated and adopted a well vascularised connective tissue structure.

The general pattern of ontogenic development of the lymphoid tissues of the turbot was similar to that reported in other teleost fish in that the thymus and kidney appeared in advance of the spleen. However, there is a marked difference in the kinetics of this development. The development of the thymus and kidney in the turbot appeared to commence at a later stage than reported in other fish. At 24h post-hatch both of these organs were in the early stages of formation, whereas, they are already well developed prior to hatching in the salmon (Ellis, 1977) and rainbow trout (Grace and Manning, 1980). That the thymus did not appear until 2 days post-hatch in the carp (Botham and Manning, 1981) may reflect the relatively short incubation time between fertilisation and hatching in this species. In the flounder, *Platichthys flesus* (Pulsford, Tomlinson, Lemaire-Gony and Glynn, 1994), the thymus could not be found in fish up to 4-6 weeks old.

At 24h, the turbot spleen was in its earliest stages of formation, in contrast with observations in many other fish species where splenic tissue appears much later (Manning, 1994). That the spleen was present at such an early stage in the turbot compared with 3 days in rainbow trout (Grace and Manning, 1980), 5 days in carp (Botham and Manning, 1981) and 42 days in salmon (Ellis, 1977), may reflect a more important role for this tissue in the developing immune system of the turbot.

These differences in the times of the first appearance and rate of development of these organs may, in part, be influenced by experimental temperature. Indeed, development of the lymphoid tissues in the Antarctic fish, *Harpagifer antarcticus*, (O'Neill, 1989) was greatly retarded in relation to the findings in other species. Although temperatures employed in that study were not

specified, it is assumed that they were relatively low. In the present study, a temperature of 16°C was employed compared, for example, to 10°C in the rainbow trout (Grace and Manning, 1980) and 4°C to 7°C in the salmon (Ellis, 1977). Nonetheless, it is difficult to equate the effect of temperature with the kinetics of lymphoid tissue development in the present study since the development of the thymus and kidney was slower than that seen in other fish with only that of the spleen advanced. If temperature alone was responsible for the differences observed, then one would expect the rate of development of each of the organs to be similarly affected.

Caution however, must be exercised when making comparisons of the effects of temperature upon such reactions in different species as the optimum will vary between species. For any real comparisons to be made, only results obtained at the optimum temperatures for the species concerned should be compared directly. Although the temperature used in the present study was close to the optimum for turbot of 15°C (Jones, 1972), the optima for the other species investigated would have to be established before direct comparisons could be made.

For this reason, development of the lymphoid tissues here was correlated with external morphological features of the larval stages which would be affected by environmental factors in the same way. Using this correlation, the stage of lymphoid tissue development could be assessed using only observations of external features of developing juvenile turbot. This may have useful implications in situations where detailed histological examination was not feasible, for example in the fish farming environment prior to vaccination.

Although at the first sampling here, the thymus and kidney had already appeared, it is the thymus which often emerges first in other fish including the salmon (Ellis, 1977), carp (Botham and Manning, 1981), rainbow trout



(Chilmonczyk, 1985; Grace and Manning, 1980), dogfish (Hart, Wrathmell and Harris, 1986), tilapia (Doggett and Harris, 1987), *Harpagifer antarcticus* (O'Neill, 1989) and sea bream (Jósefsson and Tatner, 1993). However, Chantanachookhin, Seikai and Tanaka (1991) who studied the ontogenic development of the lymphoid tissues of 3 species of marine fish found the kidney emerged first followed by the spleen and then thymus. Nakanishi (1986) reported similar findings in the rock fish, *Sebasticus marmoratus*.

Putative haemopoietic stem cells observed in the kidney of the turbot prior to the appearance of significant numbers of lymphoid cells in the thymus were also reported in the salmon (Ellis, 1977). He suggested that these cells may migrate to the thymus prior to differentiation there. Furthermore, Razquin *et al* (1990) first detected IgM-containing cells in the kidney, followed by the thymus, of the rainbow trout using anti-rainbow trout IgM monoclonal antibodies. This led them to conclude that the kidney of salmonids may be a primary lymphoid organ akin to the bone marrow of mammals. However, the findings here favour the theory that thymocytes are derived in association with pharyngeal epithelial cells. Indeed, studies using anti-thymocyte monoclonal antibodies in the carp (Secombes *et al*, 1983) demonstrated that these cells appeared initially in the thymus and thence in the anterior kidney. Furthermore, Tatner (1985) demonstrated that isotope-labelled rainbow trout thymocytes could be traced to the kidney and spleen. Jósefsson and Tatner (1993) noted that the kidney and thymus grew closer together in the sea bream and suggested that cells migrated between them. Although these organs were in close proximity in the turbot, there was no evidence for transference of cells directly between them in the present study.

## **CHAPTER 6**

### **THE RETICULO-ENDOTHELIAL SYSTEM (RES)**

#### **6.1 INTRODUCTION**

Phagocytosis, the ingestion of foreign material by single cells, is a widely utilised process, occurring throughout the animal kingdom. Phylogenetically regarded as the oldest and most fundamental defence mechanism of the host, it was first discovered over one hundred years ago by Metchnikoff in the 1880s during his investigation of the intracellular digestion of starfish larvae. To make examination of their transparent tissues easier he injected them with a carmine solution and witnessed the appearance of amoeboid cells surrounding and engulfing the carmine particles. Since these were of no nutritional value to the starfish, Metchnikoff concluded that these phagocytic cells must play some defensive role and put forward a theory that these "wandering" leucocytes formed the host's defence against invading micro-organisms. He also showed the uptake of guinea pig cells by various fish leucocytes (Metchnikoff, 1905 [reviewed by Heifets, 1982]).

At its most basic level the engulfment process is used in nutrition for the uptake of food. Single celled animals such as protozoa utilise this process as their main feeding mechanism and also as a defence mechanism. Phagocytic cells engulf infectious agents, antigenic particles and debris, internalise and then destroy them. The phagocytic system is recognised as a non-specific defence mechanism which becomes more organised with advancement up the phylogenic tree.

Phagocytosis is an integrated series of complex events which culminate in the elimination of particles. These are separated into different stages; attachment, engulfment, intracellular killing and digestion (Cohn and Hirsch, 1965). For phagocytosis to occur, contact between the phagocytic cells and

foreign material is necessary. Vascular and lymphatic systems increase the speed with which mobile phagocytic cells can reach a site of insult to deal with invading material either directly or by transporting them to the site of fixed tissue macrophages. Attachment is mediated by specific receptors on the phagocytic cell's surface to which micro-organisms must bind to initiate ingestion. Pseudopods extended to surround a particle, meet and fuse to form a phagosome (Griffin, Griffin and Silverstein, 1975; Griffin, Griffin, Leider and Silverstein, 1976). Lysosomal granules then fuse with these cytoplasmic vacuoles to form a phagolysosome, discharging their contents into it (degranulation) (Hirsch and Cohen, 1960; Hirsch, 1962). Internalised material is then digested by acid hydrolases. This whole process has been reviewed by Ryter and DeChastellier (1983).

In more advanced organisms, phagocytosis is carried out by two major categories of cells (van Furth and van Zwet, 1973). First, the smaller, non-dividing granulocytic neutrophils are largely confined to the bloodstream (unless recruited to a site of acute inflammation). Granulocytes are the dominant leucocyte type constituting 70% of circulating leucocytes in mammals. They contain a wide range of bactericidal factors and glycogen energy stores. However they are relatively short-lived, spending some 36h in the circulation before migrating through vessel walls into tissues, surviving only a few days phagocytosing invading micro-organisms (Wintrobe, 1967). The second cell type, macrophages are derived from bone marrow promonocytes and differentiate into blood monocytes (van Furth, 1970, 1975). Monocytes circulate in the blood, forming a surveillance system and settle within a variety of tissues and organs as tissue macrophages (van Furth, Dieselhoff-den Dulk, Sluiter and van Dissel, 1985). There they undergo a rapid increase in size, protein synthesis and lysosome content. Long-lived macrophages (several months in mammals) (van Furth, 1970) are metabolically active and more strongly phagocytic than monocytes, containing large quantities of rough ER and mitochondria.

Phagocytic cells organised within specific areas or organs form the basis of the reticuloendothelial system (RES). Such accumulations are strategically positioned around the body to maximise their effectiveness, filtering foreign material. The phagocytic function of the RES develops early in foetal life and once an antigen has penetrated the epithelial surface it will come into contact with these cells.

In man, the RES includes circulating cells which may be dispersed throughout the connective tissue (Kupffer cells in the liver) and cells fixed to an endothelial layer of blood capillaries as in the lung (where they are most concentrated), kidney and lining the sinusoids of the spleen and medullary sinuses. Macrophages have also been reported in the brain, alveoli, lymph nodes, bone and blood. Phagocytic activity in mammals generally appears to be most active in the liver and spleen.

Many studies have been made on the clearance and localisation of introduced foreign material in mammals. The situation has also been observed in various species of fish (Mackmull and Michels, 1932; Ellis, 1974; Ellis, Munro and Roberts, 1976; Secombes and Manning, 1980; MacArthur, Fletcher and Thomson, 1983; Hunt and Rowley, 1986a), where the spleen and the kidney have been identified as the major phagocytic organs. Although cardiac macrophages have also been shown to have some involvement (Ferguson, 1975), the roles of the gills and liver appear to be more species specific. A study of the ontogenic development of the RES in young carp (Botham, 1982) reported it to be more important in younger fish, prior to the final development of their lymphoid system.

Cells exhibiting a capacity to take up foreign material *in vitro* in the turbot have been shown to include monocytes and thrombocytes (see Chapter 3). Phagocytic monocytes have been shown in fish to migrate from blood vessels

into the tissues where they develop and reside as macrophages (Ellis, 1977). Thrombocytes, found to be phagocytic in the turbot (present study), gar (McKinney, Smith, Haynes and Sigel, 1977) and dogfish (Morrow and Pulsford, 1980) have not been identified as part of the RES. Although cells of the granulocytic series did not exhibit *in vitro* phagocytosis in turbot, they did contain enzymes and ultrastructural features concomitant with a phagocytic function. Such a phagocytic capacity has been reported as important in other fish species (Finn and Neilson, 1971; MacArthur and Fletcher, 1985) and Hine (1992) has suggested that the granulocytes of most species are capable of phagocytosis.

Since particulate antigens and inert carbon particles have been shown to be phagocytosed by the same system (Halpern *et al*, 1957, cited Botham, 1982) with particles accumulating in organs rich in phagocytes, carbon was used as the model of choice, in this study. The phagocytic system of the turbot was investigated, following the removal of foreign inert material and its ultimate fate within the tissues, thus identifying the sites of phagocytic macrophages. The uptake, localisation and clearance of colloidal carbon within the network of phagocytic tissue was visualised following an intraperitoneal injection. Histological changes in the lymphoid organs of turbot were examined at intervals from 20 min to Day 14 following injection. Phagocytic cells appeared blackened due to their carbon content when compared with non-phagocytic cells which stained normally. Tissues sampled included spleen, anterior, mid and posterior kidney, heart, blood, liver and gill.

## **CHAPTER 6**

### **6.2 MATERIALS AND METHODS**

Sections of the Materials and Methods chapter of specific relevance to this chapter include:

#### **2.3 LIGHT MICROSCOPY**

#### **2.6 PHAGOCYTOSIS**

##### **2.6.2 *IN VIVO* PHAGOCYTOSIS**

## **CHAPTER 6**

### **6.3 RESULTS**

The fate of intraperitoneally injected carbon was monitored in the turbot over a 14 day period. Anterior, mid and posterior kidney, spleen, liver, gill, heart and blood were examined at 20min, 1h, 4h, 12h, 24h, 48h, 3 days, 5 days, 7 days, 10 days and 14 days post-injection and the distribution of carbon and carbon-laden cells recorded.

Carbon introduced into the peritoneal cavity became localised within certain organs of the turbot. The spleen was the first tissue to appear blackened when examined macroscopically after injection, closely followed by the mid and posterior kidney and heart. Microscopic assessment of the relative activity of the phagocytic organs with time is summarised in Table 6.

Naturally occurring melanin, present in unbleached control sections of spleen and kidney was removed to avoid confusion with interstitial carbon particles. Sections of unbleached liver, gill and spleen all contained many more circulating cells within blood vessels and sinuses than bleached equivalents, suggesting that these may have been lost during the harsh bleaching process.

#### **6.3.1 SPLEEN**

Large aggregates of extracellular carbon were seen associated with the outer capsule of the spleen at all times throughout the study. Fine carbon deposits were first visualised within this organ 20min post-injection with sparse scattered carbon particles becoming associated with the ellipsoid walls after 1h.

By 12h there was a large increase in the amount of carbon accumulating

Table 6

DISTRIBUTION OF COLLOIDAL CARBON WITHIN MAJOR PHAGOCYtic  
ORGANS OF THE TURBOT FOLLOWING INTRAPERITONEAL INJECTION

	SPLEEN	KIDNEY		
		ANTERIOR	MID	POSTERIOR
20 min	**	*	**	***
1 h	***	**	-	-
4 h	***	***	****	***
12 h	****	****	*****	****
24 h	*****	*****	*****	****
48 h	**	**	***	**
Day 3	****	*****	*****	*****
Day 5	****	*****	****	*****
Day 7	***	****	****	****
Day 10	NT	****	***	**
Day 14	***	*****	****	NT

Symbol represents degree of phagocytic activity

- no carbon observed

phagocytic activity : \* mild to \*\*\*\*\* extreme

NT not tested

(Phagocytosed carbon also seen within blood, heart and gill interstices)



around the ellipsoids, with carbon seen inside large macrophage-like cells and the occasional MMC for the first time. Levels of carbon within the spleen rose to a maximum by 24h with phagocytes increasing in both size and number. Their intracellular loads increased as each contained fewer but larger aggregations of carbon granules. Phagocytes were present throughout the white pulp and around ellipsoids, such accumulations of cells giving rise to areas resembling new MMCs. After this time splenic levels began to fall steadily. Most existing MMCs were devoid of carbon.

A more dispersed distribution away from ellipsoids continued to Day 5 when large amounts of carbon were often seen associated with the outer walls of large blood vessels. Carbon levels decreased again by Day 7 with macrophage-like cells containing smaller amounts of carbon. At this point, some carbon was seen on the periphery existing MMCs (Figure 21a).

### **6.3.2 KIDNEY**

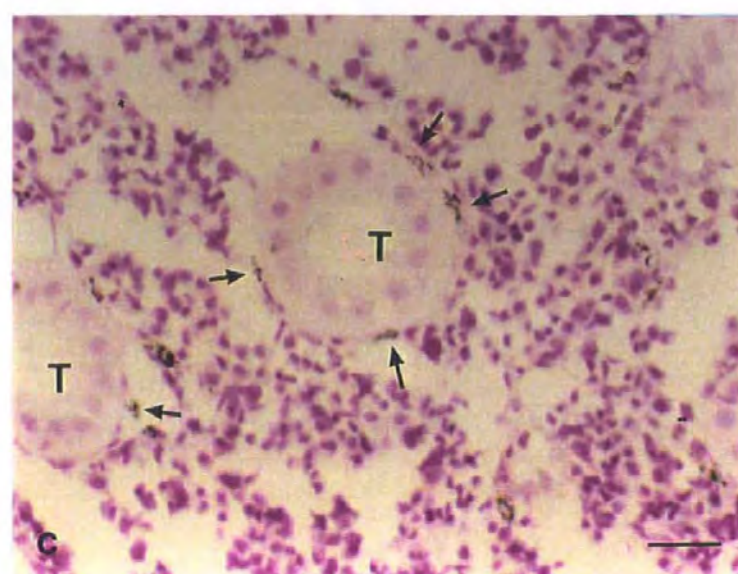
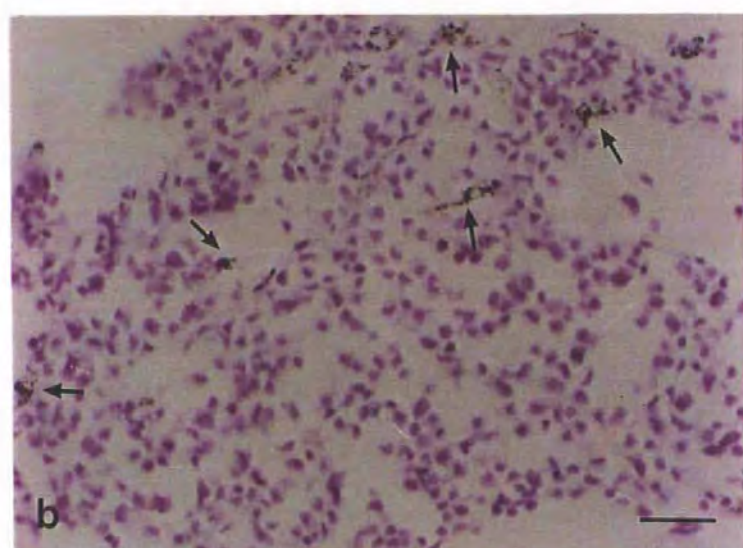
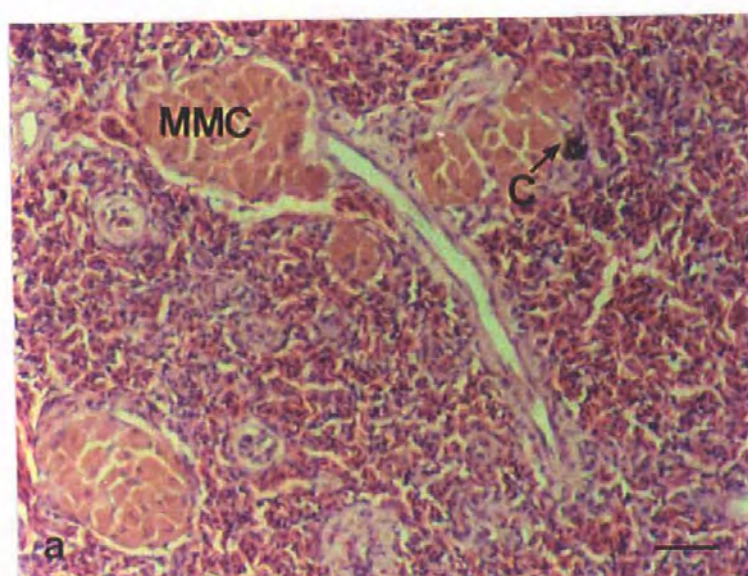
#### **6.3.2.1 Anterior Kidney**

In 20min and 1h post-injection samples, small aggregations of carbon laden cells were seen. These occurred within close proximity to renal tubules. MMCs did not appear to contain carbon at this stage. By 4h, randomly distributed large macrophage-like cells containing carbon were apparent, becoming increasingly obvious by 12h (Figure 21b) when cells containing twice the carbon loads of 1h samples were observed. MMCs remained devoid of carbon but carbon laden cells were seen aggregated into groups.

Highest carbon levels were seen after 24h with phagocytic macrophage-like cells containing even larger accumulations of carbon. By Day 3 these randomly distributed cells measured 15-18 $\mu$ m with projections extending

**Figure 21** Carbon uptake in the spleen and kidney.

- a** Spleen at 48h post injection showing carbon (C) associated with the periphery of a melanomacrophage centre (MMC). (Unbleached H&E). (Bar represents 200µm).
- b** Anterior kidney at 12h post injection. Arrows show macrophage-like cells laden with carbon. (Bleached H&E). (Bar represents 100µm).
- c** Mid kidney at 5 days post injection. Arrows show carbon-containing cells associated with kidney tubules (T). (Bleached H&E). (Bar represents 100µm).



from the cell surface. Individual phagocytic cells, heavily laden with carbon, continued to form aggregations. These cells increased in size further, reaching 20 $\mu$ m and having spiky extensions by Day 5, when they were tightly packed with larger masses of carbon. After this time, carbon levels began to fall. Large aggregations of carbon, measuring 7-15 $\mu$ m, were often seen close to existing MMCs but carbon was not seen within these centres.

By Day 14 carbon-containing cells were still randomly distributed although definite aggregations of carbon-bearing cells occurred within inter-vascular spaces. However, only very rarely were MMC's seen to contain carbon.

#### **6.3.2.2 Mid Kidney**

After 20min large carbon-containing cells were closely associated with the periphery of renal tubules. These macrophage-like cells (20 $\mu$ m) contained up to 30 carbon particles each. Cells containing small scattered granules of carbon were seen throughout the tissue by 4h post-injection. At 12h the majority of cells containing carbon were seen within the inter-tubule white pulp, with some cells carrying very heavy loads of carbon. Large aggregates of carbon also occurred within blood sinuses.

After 24h, fewer carbon-containing cells were observed but, when present, carbon occurred in larger intracellular clumps. By Day 3 the migration and grouping of individual cells heavily laden with larger carbon particles was evident. Many cells were associated with the periphery of tubules by Day 5 (Figure 21c). A reduction in carbon levels was seen from Day 7 onwards, with cells containing smaller amounts of finely scattered carbon. No carbon was observed in the MMCs of the mid kidney during the course of this study.

#### **6.3.2.3 Posterior Kidney**

At 20min post-injection, carbon was seen in aggregated masses inside blood vessels, entangled in an apparent network of fibrin and within thrombocytes.

At this time, carbon-containing cells were located around the periphery of tubules. There was little change after 1h. At 4h post-injection, a few cells containing small quantities of carbon were more randomly distributed throughout this tissue. Carbon particles were observed attached to extracellular fibres thought to have originated from fibroblasts or thrombocytes. Also evident at this time was the association of carbon with leucocytes within blood vessels, although it could not be determined whether this carbon was internalised.

The number of cells containing carbon had greatly increased by 12h post-injection, some of which had formed larger aggregations of cells. After 24h, the amount of internalised carbon had increased in many cells to form large intracellular deposits.

By Day 3 some very large cells heavily laden with carbon were observed (Figure 22a). These were mostly, although not exclusively, associated with the tubules. At Day 5, heavily laden cells were widely distributed throughout the tissue and from Day 7 the number of cells containing carbon decreased, although the quantity of carbon contained within each cell remained high.

### **6.3.3 HEART**

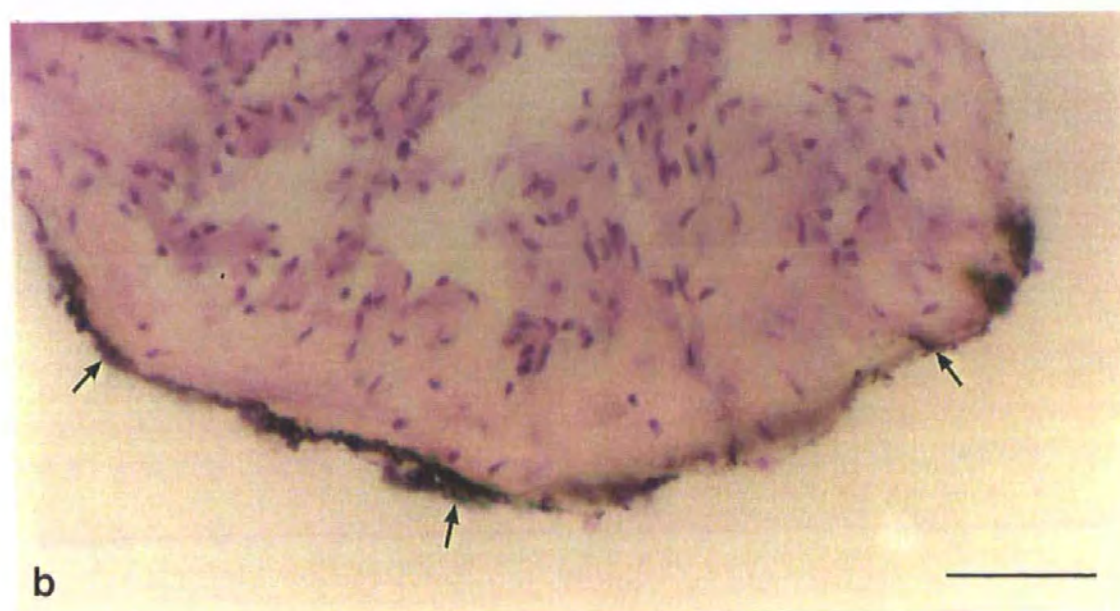
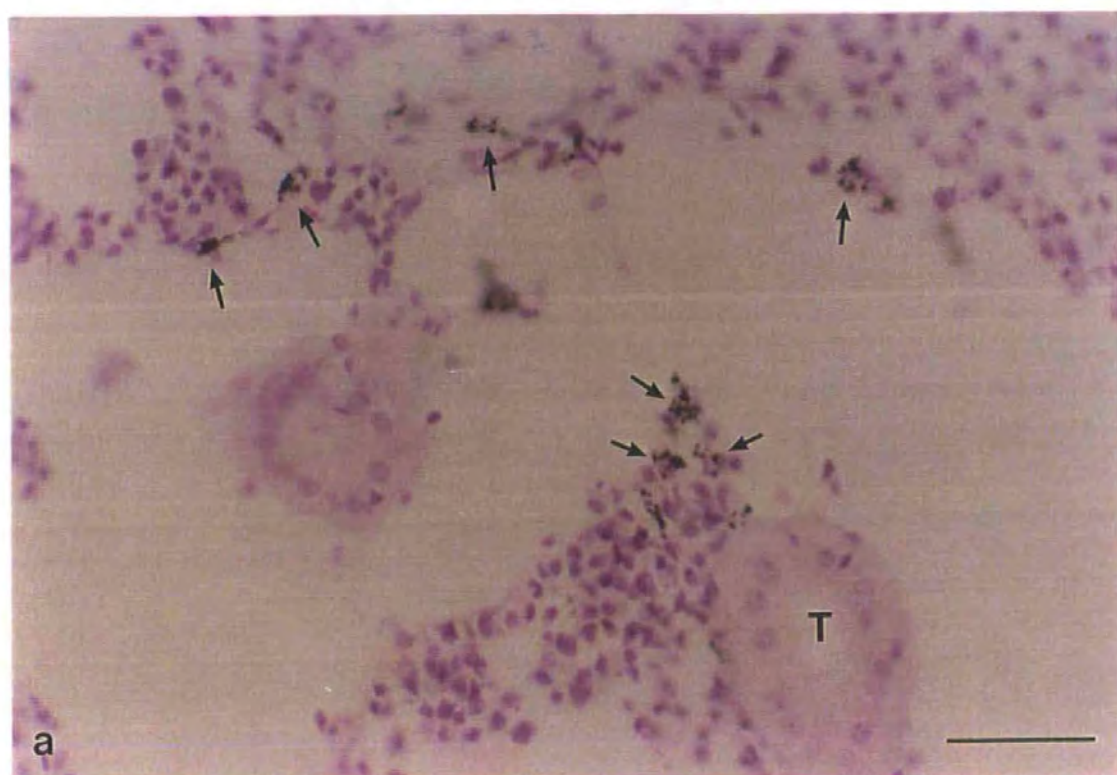
Both extracellular and internalised carbon was seen within the blood of vessels of the heart from 1h to Day 10. Uptake of carbon by phagocytic cells was evident within the musculature of the atrium at 4h post-injection. Leucocytes containing ingested carbon were also observed at this time within a large blood clot. Carbon uptake within the musculature of the atrium increased to a maximum level at Day 10 but always remained at a relatively low level. Macrophages, heavily laden with carbon, were evident in the ventricular epicardium (Figure 22b) from 1h and throughout the experimental period.

**Figure 22    Carbon uptake in the posterior kidney and heart.**

- a            Posterior kidney at 3 days post-injection showing macrophage-like cells heavily laden with carbon (arrows). Kidney tubule (T). (Bleached H&E).**
  
- b            Heart at 12h post-injection showing large quantities of carbon (arrows) associated with the epicardium. (Bleached H&E).**

**(Bars represent 200 $\mu$ m)**





#### **6.3.4 BLOOD**

At 20min small amounts of free carbon were seen in the bloodstream. A high incidence of cytoplasmic vacuolation was observed in thrombocytes, granulocytes and monocytes. Fine particles of carbon were apparent within the cytoplasm of some monocytes. At 1h, small amounts of free carbon were still present. Monocytes had a more highly vacuolated appearance than other leucocyte types. Fine scattered carbon particles were seen associated with the surfaces of thrombocytes, very occasionally appearing internalised. Large numbers of thrombocytes in blood samples were lysed. Although vacuolation of monocytes and granulocytes had increased by 4h, internalised carbon was minimal and only observed in monocytes. Increased numbers of larger, seemingly immature granulocytes were seen.

All free carbon had been cleared from the bloodstream by 12h, when leucocyte vacuolation was reduced and only one heavily laden monocyte was seen. Thrombocytes were mostly negative, only rarely seen to contain very small quantities of carbon. Granulocytes with bilobed nuclei were more frequently encountered at this time. Although granulocytes exhibited reduced cytoplasmic vacuolation, limited numbers now appeared to contain very fine particles of scattered carbon. Carbon was still found within monocytes at 48h and by Day 3 an increase in the number of monocytes and granulocytes laden with carbon was seen. A granulopenia seen at Day 5 had subsided somewhat by Day 7 when no carbon was seen within granulocytes which by now showed only marginal vacuolation. By Day 14 leucocyte morphology and numbers appeared to have returned to their pre-injection state.

#### **6.3.5 LIVER**

At 20min post-injection, particles of extracellular carbon and cells containing carbon were seen within the blood vessels and sinuses of the liver. From 4h onwards carbon occurred at the periphery of the organ, associated with the capsule but no phagocytic activity was seen within the liver itself.



### **6.3.6 GILL**

Carbon-bearing leucocytes were seen within the capillary lumen of the primary and secondary lamellae of the gills from 1h to Day 3. No apparent phagocytic activity was observed within the gill itself.

## CHAPTER 6

### 6.4 DISCUSSION

The phagocytic activity of the RES of the turbot has been demonstrated here using carbon. Its uptake in the turbot following *ip* injection was rapid, with free carbon particles observed in the blood after 20min. The subsequent clearance of carbon from the blood was equally rapid, with the majority of free carbon appearing to have been removed by 12h post-injection, with only limited amounts of carbon being seen within the blood of turbot at any time. Similar kinetics of uptake and removal of carbon in the blood have been reported in the carp (Botham, 1982) and the plaice (Ellis, 1976). The removal of particulate material from the bloodstream of fish is composed of an initial rapid phase followed by a slower one (Avtalion, 1981). Ferguson, Claxton, Moccia and Wilkie (1982) found 90% of bacteria was cleared from the blood of rainbow trout by 15min. The small amounts of carbon seen here may represent the limited amount of carbon not taken up as part of a rapid first-phase clearance.

Mackmull and Michels (1932) suggested that carbon uptake following intraperitoneal injection in the cunner, *Tautogalabrus adspersus*, was via the mesenteric blood vessels, a view supported by Ellis (1976). The presence of large numbers of lysed thrombocytes early in the response of the turbot and the association of carbon with the resulting fibrous network as well as the surfaces of intact thrombocytes, may provide a mechanism whereby free carbon particles are removed from the circulation. However, it is possible that such lysis may simply have been stress induced. Low levels of intracellular carbon were seen within vacuolated thrombocytes and monocytes in the turbot up to 12h post-injection, with an increased number of granulocytes appearing to become involved by Day 5. However, there was an apparent absence of significant numbers of cells containing phagocytosed

carbon in the blood at early time intervals post-injection and a relatively late peaking of granulocyte numbers when tissue levels had already reached the maximum level observed. This suggests that carbon was transported in the blood as free particles and removed by fixed phagocytic cells in the various organs of the turbot and supports similar findings in the plaice (Ellis, 1976) and in the carp (Botham, 1982).

The phagocytic capability of the turbot was shown here to be closely associated with the lymphoid tissues, namely the spleen and kidney, although some involvement of the heart was also seen. It has been demonstrated that these are the major phagocytic tissues in other teleost species (Wisloski, 1917; Mackmull and Michels, 1932; Ferguson, 1976; Ellis, 1976 and Botham, 1982).

Splenic ellipsoids were the first site of phagocytosis of carbon in the turbot as the particles were filtered from the blood on their passage through the organ. This process occurred rapidly, with carbon being present in the spleen after just 20 min. These findings support those of Ferguson (1976) who reported a tendency for foreign material to accumulate within ellipsoids prior to it being transferred to the MMC's. Like the mammalian spleen (Stolinsky, 1937), that of fish is known to have phagocytic cells surrounding the ellipsoids (Ellis, 1976) and represents the major site of removal of particulate debris, including effete erythrocytes from the blood. Secombes and Manning (1980) showed splenic ellipsoids to be the site of initial entrapment of carbon and particulate antigens in the carp. Botham (1982) further supported their findings in this species. The findings here support those of Ellis (1976) who put forward three main routes of carbon uptake in the spleen of the plaice, namely via the phagocytic cells in the ellipsoid sheaths, by passage through the terminus of ellipsoids into the red pulp and by entry from the blood of macrophages containing phagocytosed carbon.

The relative depletion of carbon from the ellipsoids in the latter stages of the response in the turbot coincided with the accumulation of carbon within phagocytic cells within the splenic pulp, suggesting a migration of replete macrophages through the tissues. However, few of these cells became associated with existing MMC's but instead formed new cell aggregates resembling new MMC's. In contrast, Ferguson (1976) suggested that cells migrated from the ellipsoids to existing MMCs in the spleen of turbot. The results here also contrast with those in the plaice (Ellis, 1976) and the cunner (Mackmull and Michels, 1932) but are similar to the findings of Botham (1982) who also reported minimal carbon levels in existing MMC's of the carp.

These species differences may reflect alternative methods for processing particulate material. Indeed, the formation of new MMC's is known to occur in response to antigenic challenge (Agius, 1979) and it may be that the aggregates of carbon-containing cells seen here in the turbot spleen would have formed MMC's in due course. However, in both cases, it is possible that aggregation of carbon-containing cells at the MMC's may have occurred beyond the time of the termination of the experiment. Furthermore, it is possible that the MMC's of the turbot are specific for particular materials and therefore carbon would be precluded from existing centres established to process alternative foreign matter.

The kidney was the second major site of carbon uptake in the turbot. Much work has identified the kidney as one of two major organs of the RES in fish (Ellis *et al*, 1976; Chilmonczyk and Monge, 1980; MacArthur *et al*, 1983; Imagawa, Hashimoto, Kon and Sugimura, 1990). In the investigation of the clearance rate of carbon and red blood cells (RBCs) in the plaice, MacArthur *et al*, (1983) found that the kidney was responsible for the phagocytosis of twice the amount of RBCs as the spleen. They found that uptake was not simply a function of the anatomical arrangement of organs in relation to the iv

injection site, since the kidney (the first organ to be encountered) still took up more foreign antigen when exposure was via the *ip* route. They also found that although the kidney was the main phagocytic organ, uptake rates indicated the phagocytic cells of the spleen were more active.

In the turbot, carbon-containing cells were first observed in the reticulo-endothelial (RE) cells which formed a network throughout the parenchyma of the kidney and were particularly evident surrounding the nephric tubules. Similar findings were reported in the carp (Botham, 1982) and the plaice (Ellis, 1976).

By the final sampling interval (Day 14), carbon-containing phagocytes were beginning to form aggregates within lymphoid areas of the kidney. However, as in the spleen, no significant association of carbon with existing MMC's was observed here. In contrast, migration of carbon-replete macrophages to the MMC's was reported the cunner (Mackmull and Michels, 1932) and in the plaice (Ellis, 1976).

Although the spleen and kidney were responsible for clearing the majority of carbon, some active phagocytosis was seen in the heart of the turbot supporting the findings of Ferguson (1975) who described phagocytosis in the plaice heart. Macrophages, heavily laden with carbon were initially observed within the ventricle epicardium and latterly within endocardial lining cells of the atrium. However, aggregation of carbon-containing cells was not evident here. This is in keeping with carbon uptake by the heart of the plaice (Ellis, 1976) and the carp (Botham, 1982). Indeed, the heart has often been regarded as having a highly phagocytic capability, particularly in the cunner (Mackmull and Michels, 1932), where it was thought to represent a key part of the RE system. Ellis (1976) suggested that replete phagocytes of the heart rounded up and passed to the lymphoid tissues via the

circulation, a mechanism suggested by Ferguson (1975) to help protect this organ from infection.

In keeping with the findings in other teleost species (Mackmull and Michels, 1932; Ellis, 1976; Botham, 1982), the turbot liver does not appear to play a role in the filtration and clearance of particulate matter. In contrast, the mammalian liver is known to contain large numbers of highly phagocytic Kupffer cells which remove up to 90% of injected carbon. Furthermore, the liver of the dogfish appeared to be intensely phagocytic (Hoskins and Hoskins, 1918) and was the only organ in this species to demonstrate aggregation of phagocytes. The reduced filtering capacity of the turbot liver, with its smaller number of sinuses may be responsible for it requiring fewer phagocytic cells and hence failing to take up carbon.

Although free carbon and carbon-containing cells were seen within the gills of the turbot there was no evidence of phagocytic activity. This is in keeping with the findings in most teleosts. However, Chilmonczyk and Monge (1980) demonstrated the phagocytic activity of gill pillar cells 2h post-injection, linking pillar cell function to that of MMC's. Furthermore, the dogfish gill was shown to be a site of marked phagocytosis (Hoskins and Hoskins, 1918), as was the gill of the lamprey (Yamaguchi *et al*, 1979).

The identity of the RE cells in the turbot remain uncertain. However, a role for fibroblasts cannot be ruled out as fibrous cells were observed in association with carbon particles in the present study. Mackmull and Michels (1932) claimed that connective tissue fibroblasts of the cunner were phagocytic. However, Ellis (1976) was unable to confirm whether the phagocytic cells of the RE system in the plaice were fibroblasts and suggested a more likely role for macrophages. Alternatively, thrombocytes, which have been shown in this study to be highly phagocytic *in vitro*, may

form part of the RE system in the turbot but further studies would be required to confirm the identity of these cells in the turbot.

## CHAPTER 7

### *IN VITRO* LEUCOCYTE MIGRATION

#### 7.1 INTRODUCTION

Leucocyte migration is an integral part of many biological processes occurring under normal and pathological conditions (Varani, 1985). The attraction of cells into regions of infection and inflammation to remove foreign substances and dispose of damaged tissue is essential for host defence. Such directed migration is thought to account for leucocyte accumulation at sites of injury in response to chemical gradients of chemotactic factors produced or present within infected areas (Snyderman and Mergenhagen, 1976; Wilkinson, 1984). In mammals these chemotactic factors, derived from various sources including fungi, bacteria and their growth products, complement, clotting systems and cells (reviewed by Zigmond, 1978) bind to specific cytoplasmic receptors of neutrophils inducing chemotactic locomotion. The molecular and cellular basis of such locomotion has been reviewed (Snyderman and Goetzel, 1981; Keller and Till, 1983; Wilkinson, 1984).

In the absence of a chemical stimulus, cells migrate slowly and randomly (Wilkinson, 1976). When present, chemical attractants can influence both the rate and / or the direction of cell migration, resulting in directed migration. Chemotaxis, chemokinesis and directed migration are all terms which exist to describe the different types of response. To avoid confusion and understand the behaviour of cells in an inflammatory situation, it is necessary to distinguish clearly between them (Wilkinson, 1985). Chemotaxis is a reaction by which the direction of locomotion of cells is determined by substances in their environment. Migration can be both positive (towards gradient source) or negative (away from source). Chemokinesis is a reaction by which the speed or frequency of locomotion



and / or the frequency and magnitude of turns of cells is determined by substances in their environment (Wilkinson and Allan, 1978). Increased rates of migration will not necessarily result in cell accumulation just because the cells will be moving faster, as once at the source there is nothing to prevent them moving away again. Directed migration is not always synonymous with chemotaxis, it may be chemotactic but it can also result from contact guidance (Weiss, 1959). This phenomenon can be observed within an haemocytometer as cells accumulate with time along the etched grooves of the chamber.

Chemotaxis represents the end result of a complex series of events which include; the binding of chemotactic attractants to specific cell surface receptors, phospholipase activation and arachadonic acid release, and morphological polarisation of cells (Cianciolo and Snyderman, 1982). Reorientation of internal structures of cells prior to changes in direction of locomotion suggests that the orientation process is critical for and not merely a consequence of directional migration.

Ultrastructural analysis of mammalian leucocytes during cell migration has revealed that neutrophils rapidly assume a highly organised and oriented morphology (Malech, Root and Gallin, 1977). The first event to occur is the appearance of a veil-like flattened hyaline membrane or lamellipodium at the anterior end of the cell which may show considerable ruffling. Cytoplasmic organelles remain in the more sharply delineated posterior portion of the cell. There may be a tail (with or without reticulin fibres) and the nucleus is positioned towards the rear of the cell with the bulk of the cytoplasm and granules between it and the advancing pseudopodia. Microtubules, localised in assemblies at the leading end of the cell, are thought to be important in maintaining cell orientation. This orientated morphology is retained during locomotion, with the cell elongating and cytoplasmic contents streaming forwards into the anterior part of the cell. This has been described for

mammals by Wilkinson and Allan (1978). Such ultrastructural changes have also been demonstrated in shark leucocytes during chemotactic migration (Hyder-Smith, Obenauf and Smith, 1989).

Zigmond, (1974, 1977) observed that cells became orientated toward a gradient source before translocation was begun, suggesting the ability of a cell to compare concentrations of an attractant at different points along its own surface and detect a gradient across its length. It was concluded that this sensing system must be spatial rather than temporal since orientation of the cell by sampling gradients at different times would require cell movement to detect the gradient, which is not the case.

For cells to respond directionally to a stimulus they must possess sites capable of recognising the stimulus. Chemoattractant ligands bind to specific cell surface receptors producing the first step of activation for directed locomotion (Snyderman and Pike, 1984). Many factors are known to stimulate mammalian leucocyte migration including; bacterial metabolites, serum factors, cell derived materials from stimulated lymphocytes and neutrophils and denatured proteins. Supernatants from cultures of rapidly growing bacteria were shown to contain potent chemoattractants (Keller and Sorkin, 1967). Bacterial culture supernatants, known by Metchnikoff to possess chemotactic activity as long ago as 1888, were shown to contain protein and lipid components.

The use of synthetic N-formyl methionyl peptides as experimental chemotactic agents was based on the observation that products of bacteria, unlike eukaryotic cells, initiate protein synthesis with an N-formyl methionyl group (Schiffmann, Corcoran, and Wahl, 1975). This led to the suggestion that such peptides, capable of inducing chemoattraction, could be recognised by host leucocytes as "non self". Of the synthetic formyl peptides, analogues of the bacterial peptides present in bacterial secretions shown to attract

phagocytes, N-formyl methionyl-leucine-phenylalanine (FMLP) is the most potent so far synthesised (Freer, Day, Radding, Schiffmann, Aswanikumar, Showell and Becker, 1980; Snyderman and Goetzl, 1981; Marasco, Pham, Krutzsch, Showell, Feltner, Nairn, Becker and Ward, 1984).

Chemotactic activity has been reported in response to serum (Boyden, 1962). The finding that heating serum to 56°C for 30min prevented this activity led him to suggest the involvement of complement. Activation of serum results in enzymatic splitting of serum proteins C3 and C5 to produce strong mammalian chemotaxins. C5a generated during the activation of complement is the major chemotactic peptide for leucocytes (Damerau, Zimmerman, Grünfeld, Czorniak and Vogt, 1980). It is important since it can be generated via the classical or alternative pathways at the surface of bacteria and other cells even though such cells are not themselves releasing chemotactic factors.

Casein has been shown to be a very potent chemoattractant (Keller and Sorkin, 1976) attracting mammalian neutrophils and macrophages. It is not a single protein, but a complex of  $\alpha$ ,  $\beta$  and  $\kappa$ -caseins. Structurally similar  $\alpha$  and  $\beta$ -caseins are both chemotactic but little or no activity has been reported to  $\kappa$ -casein (Wilkinson, 1974).

Lipid molecules including prostaglandins are known to be important as chemotactic factors (Turner, Campbell and Lynn, 1975). Bacterial culture filtrates are another source of chemotactic lipid and lipid-like materials. A group of non-bacterial lipids involved in inflammation, the Leukotrienes, are a series of closely related compounds derived from arachadonic acid. Of the phagocyte derived metabolites of arachadonic acid, Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) is an extremely potent chemoattractant, producing a rapid accumulation of leucocytes when given locally *in vivo* (Turner and Lynn, 1978; Goetzl and Pickett, 1980).

Migration of fish leucocytes *in vitro* has been demonstrated in a number of species utilising each of the assays tested here. These include the rainbow trout, (Griffin, 1984; Sharp *et al*, 1991), nurse shark, *Ginglymostoma cirratum*, (Obenauf and Hyder Smith, 1985), plaice (MacArthur *et al*, 1985; Nash *et al*, 1986), eel, *Anguilla japonica* (Suzuki, 1986), spot, *Leiostomus xanthurus* and hogchoker, *Trinectes maculatus*, (Weeks, Warinner, Mason and McGinnis, 1986), carp, (Howell, 1987) and eel (Iida and Wakabayashi, 1988). Weeks, *et al* (1986) demonstrated an impaired chemotactic responsiveness in fish taken from polluted rivers and Sharp *et al* (1991) found an increased chemokinetic response to parasitic antigens.

Currently available assays which demonstrate *in vitro* cell locomotion in the presence of attractants fall into two categories. Firstly, characterisation of the migration of a proportional sample of the whole cell population and secondly, direct observation of the locomotory activity or shape of individual cells. Both have advantages and disadvantages and give information on different aspects of the response. Such methods include the Under Agarose Migration Assay (Cutler, 1974; Nelson, Quie and Simmons, 1975), Millipore Filter Assay system (Boyden, 1962), Capillary Tube Migration Assay (George and Vaughan, 1962; David, Al-Askari, Lawrence and Thomas, 1964) and direct observation of the shape of individual leucocytes in the Leucocyte Polarisation Assay (Haston and Shields, 1985).

In the "Under Agarose" technique, leucocytes placed into the central well of three, migrate beneath an agarose layer towards a test attractant or control solution placed in the outer wells. With no attractant present, migration of cells from the central well is circular, whereas in the presence of an attractant migration is distorted. Comparison of active cell migration towards the attractant with spontaneous migration towards the control, allows a Chemotactic Index (CI) to be determined within the same cell population for each attractant.

Filter assays contain two chambers, separated by a filter. A standardised cell suspension is placed into the upper compartment and the chemotactic or control substance into the lower. Cell migration through pores in the filter occurs in response to the stimulus beneath.

Two types of filter are commonly used. Firstly, the thicker nitro-cellulose filter (100-150 $\mu$ m) allows assessment of migration of separate cell populations within the filter (since migration is usually stopped before cells completely traverse the filter). Measurements of migration are then made at different distances down through the filter using fine adjustment on a microscope. Secondly, the much thinner polycarbonate filter (10 $\mu$ m), with its shorter incubation times, only facilitates cell observation and counts to be made on one or other of the filters surfaces. Treatment of the filter with polyvinylpyrrolidone (PVP) prevents migrated cells being lost from the underside. Use of the polycarbonate filter in a microchemotaxis chamber instead of a standard chamber can reduce the volumes of chemoattractant required (from 0.5ml to 100 $\mu$ l), thereby reducing incubation times, and is easier to quantify.

In the Capillary Tube Migration Assay, leucocytes are packed into capillary tubes which are placed in a solution of test substance. Migration of cells from the open end of the tube is quantified by direct measurement and used as an indication of the ability of the substance to induce cell locomotion.

Assays which rely on direct observation of individual cells include the Leucocyte Polarisation Assay which is based upon the observation that cell orientation, one of the earliest responses to a chemoattractant, is a prerequisite for locomotion (Cianciolo and Snyderman, 1981). Zigmond, Levitsky and Kreel (1981) reported that leucocytes take up a polarised morphology in response to uniform concentrations of chemotactic factors.

When placed in a suspension of an homologous attractant solution the cells can be observed to change from their normal, non stimulated spherical appearance to an elongated, polarised shape when activated (Smith, Hollers, Patrick and Hassett, 1979; Keller, Naef and Zimmermann, 1984; Haston and Shields, 1985). A high correlation between polarisation and chemotactic response has been reported (Cianciolo and Snyderman, 1981). Responses can be rapid and within the first minute up to 75% of cells capable of polarisation will have done so, with maximum polarisation levels being reached after 20-30min (Cianciolo and Snyderman, 1982).

In the present study a variety of endogenous and exogenous factors, previously shown to be chemotactic/chemokinetic for mammalian and fish leucocytes were tested and several techniques employed to find a sensitive and easily reproducible assay able to consistently detect turbot leucocyte migration *in vitro*.

## **CHAPTER 7**

### **7.2 MATERIALS AND METHODS**

Sections of the Materials and Methods Chapter of specific relevance to this chapter are:

#### **2.1 FISH**

#### **2.2 HAEMATOLOGICAL STUDIES**

##### **2.2.6 BLOOD AND TISSUE LEUCOCYTE PREPARATION**

#### **2.7 PREPARATION OF CHEMOATTRACTANTS**

##### **2.7.1 BACTERIAL CULTURE FILTRATES**

##### **2.7.2 N-FORMYL METHIONYL-LEUCYL-PHENYLALANINE (FMLP)**

##### **2.7.3 SERA**

##### **2.7.4 CASEIN**

##### **2.7.5 LEUKOTRIENE B<sub>4</sub>**

#### **2.8 ASSAYS OF CELLULAR RESPONSES**

##### **2.8.1 UNDER AGAROSE MIGRATION ASSAY**

##### **2.8.2 MICROCHEMOTAXIS CHAMBER ASSAY**

##### **2.8.3 CAPILLARY TUBE MIGRATION ASSAY**

##### **2.8.4 LEUCOCYTE POLARISATION ASSAY**

## CHAPTER 7

### 7.3 RESULTS

Turbot leucocyte migration was assessed to *Vibrio alginolyticus* culture supernatant, synthetic bacterial peptide FMLP, normal and zymosan activated turbot serum, LTB<sub>4</sub> and casein using the under agarose migration assay, microchemotaxis chamber, capillary tube migration and polarisation assays.

#### Statistical Analysis

The Student's t-test was used to determine the significance of differences between experimental results. A significant difference was given where  $p \leq 0.01$  when compared with the relevant control.

#### 7.3.1 UNDER AGAROSE MIGRATION ASSAY

##### 7.3.1.1 Comparison of Assay Conditions

Preliminary investigations detected large variations in migration and a lack of reproducibility of results. In order to optimise test conditions, several variables were investigated; migration surface, agarose type and agarose concentration. When a variety of commercially available tissue culture grade petri dishes (see Section 2.8) and glass microscope slides coated in gelatine were compared, the substrate upon which consistently high quality migration patterns were observed was gelatine-coated glass microscope slides. Migration distances were greater using Agarose type IV than Agarose type I. The percentage of agarose present in gels (1.5% - 0.5%) had the greatest effect of the variables tested. Migration distances increased as the concentration of agarose decreased, being maximal at 0.5%. However, gels became progressively more difficult to dry out and manipulate as their liquid content increased, resulting in loss of migrated cell patterns. Thus, optimal experimental parameters for this technique which were adopted here, used



concentrations of 0.8% type IV agarose poured onto gelatine-coated glass microscope slides.

### **7.3.1.2 Comparison of Chemoattractants**

Responses were measured to various concentrations of a 24h culture supernatant of *V. alginolyticus*, normal and zymosan activated turbot serum, FMLP and casein. Cellular migration was expressed as the Chemotactic Index (CI), the ratio of cell movement towards the attractant to that in the direction of the control. (CI=1 random migration, CI <1 inhibition, CI >1 attraction). Chemotactic Indices for turbot leucocytes are summarised in Table 7.

The CI's of cell movement towards normal and activated serum were not significantly different.

At concentrations of *V. alginolyticus* culture supernatants of 40% and above, marked inhibition of migration was consistently observed. The inhibitory effects of a 100% bacterial culture supernatant can be clearly seen in Figure 23. The degree of inhibition decreased and CIs became more variable as the bacterial culture filtrate was diluted (Table 7).

No directional migration was detected in response to the presence of either casein or FMLP at any concentration tested.

### **7.3.2 MICROCHEMOTAXIS CHAMBER ASSAY**

Responses were measured to various concentrations of a 24h culture supernatant of *V. alginolyticus*, normal, heat inactivated, zymosan activated and zymosan control turbot serum, LTB<sub>4</sub>, FMLP and casein. Cell migration was measured as the number of cells moving through the pores to the underside of the filter in response to the test attractant beneath, when counted

Table 7

CHEMOTACTIC INDICES OF TURBOT LEUCOCYTES IN RESPONSE TO A VARIETY OF CHEMOATTRACTANTS USING THE UNDER AGAROSE ASSAY

ATTRACTANT	CHEMOTACTIC INDEX (CI)	
<i>Vibrio alginolyticus</i>		
0.1%	1.12 (± 0.35)	
1.0%	1.11 (± 0.30)	
10%	0.88 (± 0.02)	
25%	1.28 (± 0.48)	
40%	0.84 (± 0.09)	*
50%	0.75 (± 0.18)	*
100%	0.50 (± 0.19)	*
Normal serum	1.04 (± 0.08)	
Activated serum	1.19 (± 0.43)	

Chemotactic indices (CI) (± SD) represent a ratio of migration in direction of test substance (A) to migration towards control (B), ( $A/B$ ).

Figures were calculated following 3h incubation at 20°C.

(Distances represented mean average of a minimum of 3 observations from each of 5-7 fish).

\* indicates significant inhibition ( $p \leq 0.01$ )

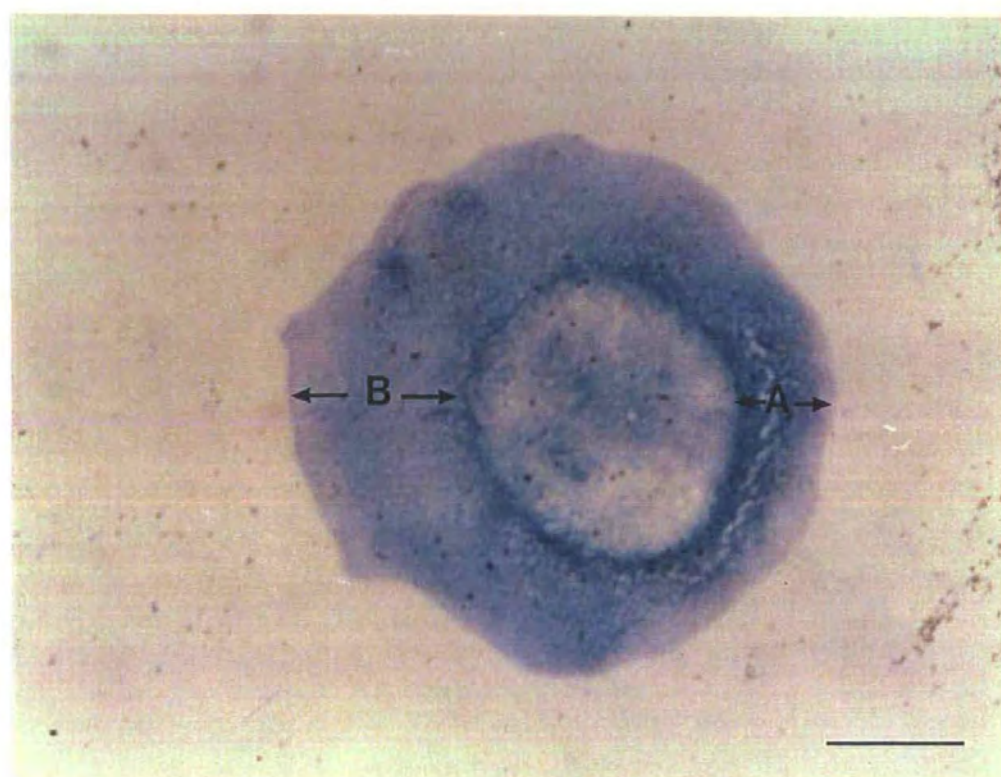
**Figure 23** Effects of *Vibrio alginolyticus* culture supernatant on leucocyte migration in the under agarose assay.

Differential migration pattern of turbot kidney leucocytes incubated for 3h with 100% *Vibrio alginolyticus* culture supernatant.

**A** indicates migration in direction of bacterial supernatant.

**B** indicates migration towards control supernatant.

(Bar represents 1mm)



from 10 high power fields of view (HPF, x400 magnification). The majority of migratory turbot leucocytes observed on the underside of filters had the appearance of granulocytes (Figure 24).

*V. alginolyticus* culture supernatants were seen to be capable of inducing significantly enhanced migration above that of controls at concentrations between 5% and 50% being maximal at a concentration of 20% (Figure 25). The highest of migrating cells per HPF was observed in response to this attractant when compared with any of the others tested.

High levels of migration were seen in response to normal, heat inactivated, zymosan activated and zymosan control sera (Figure 26). The strongest migratory responses were induced by normal serum at concentration greater than 50%. At concentrations lower than 50%, there was no significant difference between migratory response to normal serum and the other sera tested. However, migration in response to zymosan activated serum was significantly higher than to zymosan control serum at concentrations between 1% and 50% but not at 75% or 100%.

Significant migration occurred in response to  $\text{LTB}_4$  at concentrations of  $10^{-7}\text{M}$  and  $10^{-6}\text{M}$  and were greatest at the latter concentration (Figure 27). Cellular migration levels did not rise above those seen for the control at any other concentrations tested.

No significant migration of leucocytes was detected in the presence of casein or the synthetic bacterial peptide analogue FMLP when compared with controls ( $p \leq 0.01$ ).

Figure 24    Migratory leucocytes in the microchemotaxis chamber assay.

Turbot kidney leucocytes on the underside of a filter in response to Leukotriene B<sub>4</sub> (10<sup>-7</sup>M).

- G    neutrophil-like granulocyte
- P    indicates a 3µm filter pore

(Bar represents 10µm)

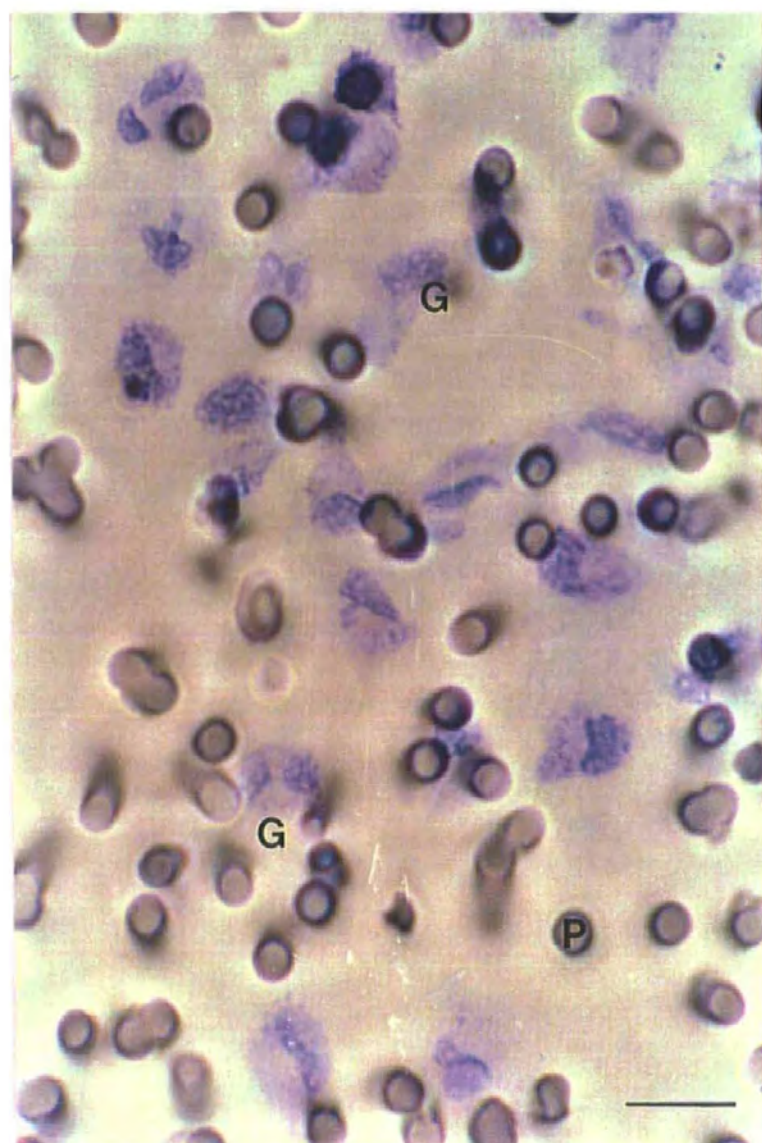


Figure 25    Effects of *Vibrio alginolyticus* culture supernatant on leucocyte migration using the microchemotaxis chamber.

The effect of various concentrations of a 24h *Vibrio alginolyticus* culture supernatant (●) and control supernatant (O) on turbot kidney leucocyte migration.

Symbols represent average number of migrated cells observed on underside of filter per 10HPFs in triplicate assays in each experiment

( | indicates  $\pm$ SE obtained from 5 fish)

Significantly different points are marked \* ( $p \leq 0.01$  compared with control).



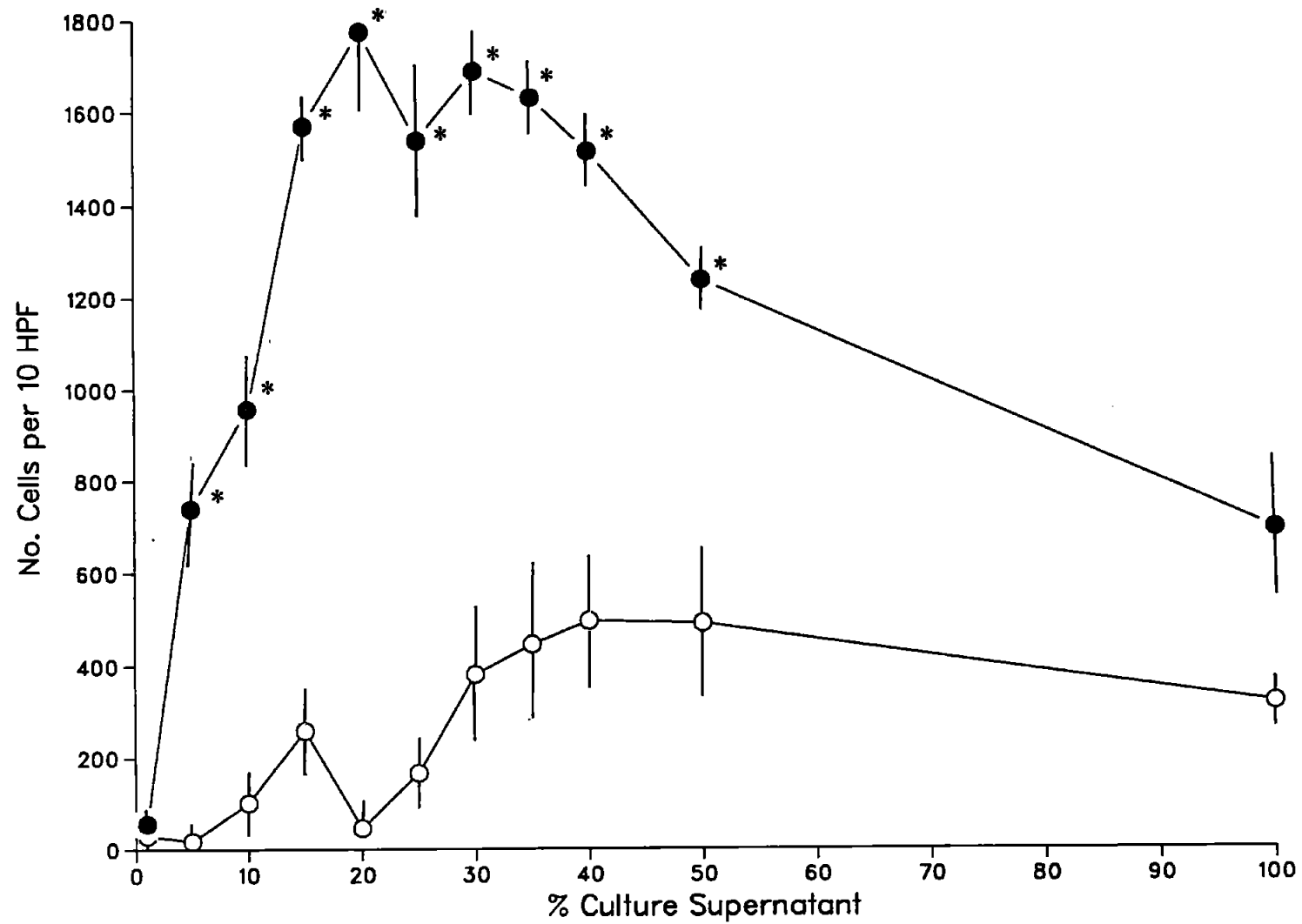


Figure 26 Effects of serum on leucocyte migration using the microchemotaxis chamber.

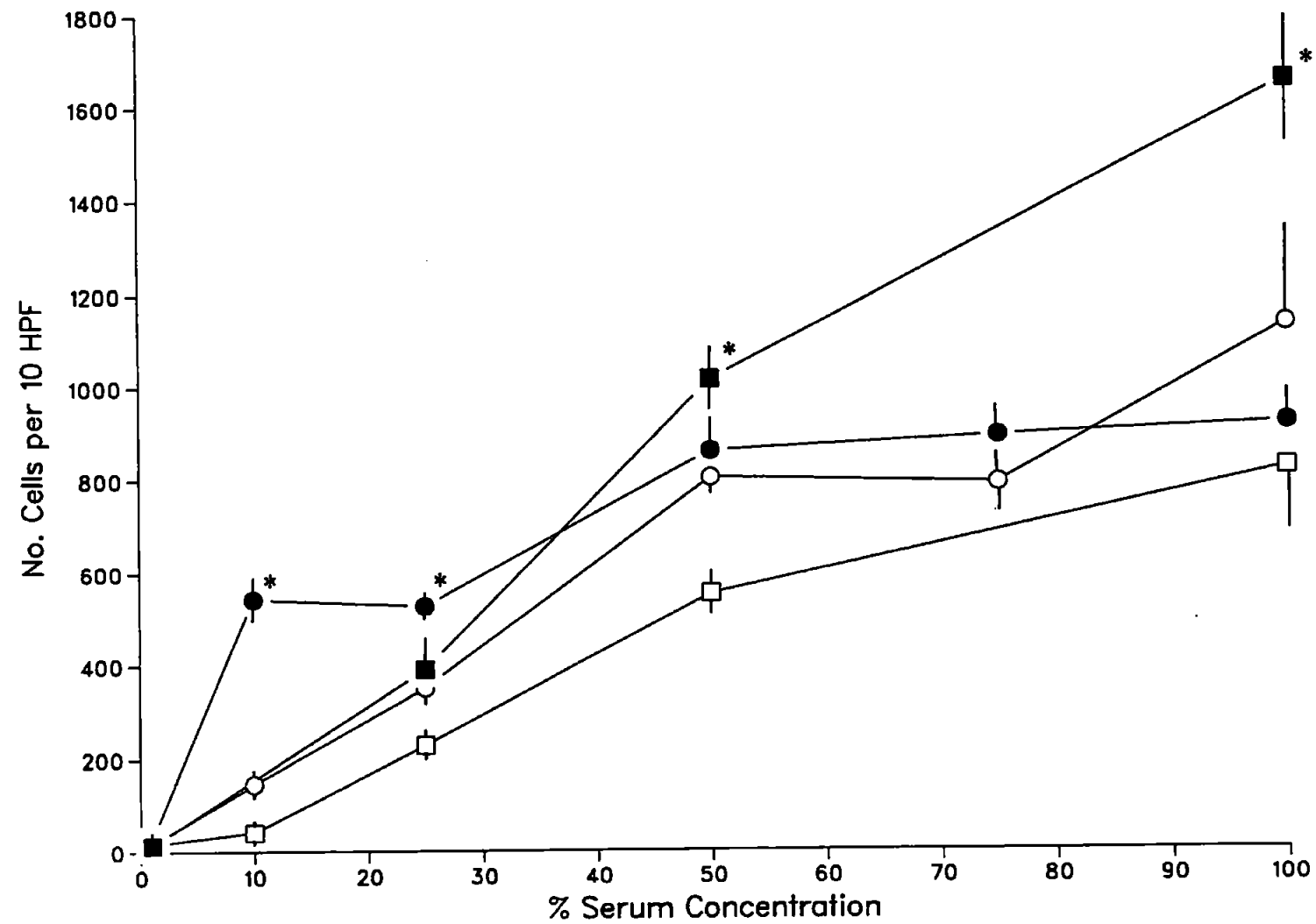
The effect of various concentrations of normal turbot serum (■), heat inactivated normal serum (□), zymosan activated serum (●) and zymosan control serum (○) on turbot kidney leucocyte migration.

Symbols represent average number of migrated cells observed on underside of filter per 10HPFs in triplicate assays in each experiment

( | indicates  $\pm$ SE obtained from 5 fish)

For statistical analysis, normal serum (■) was compared with heat inactivated normal serum (□) and zymosan activated serum (●) was compared with zymosan control serum (○)

Significantly different points are marked \* ( $p \leq 0.01$  compared with control).



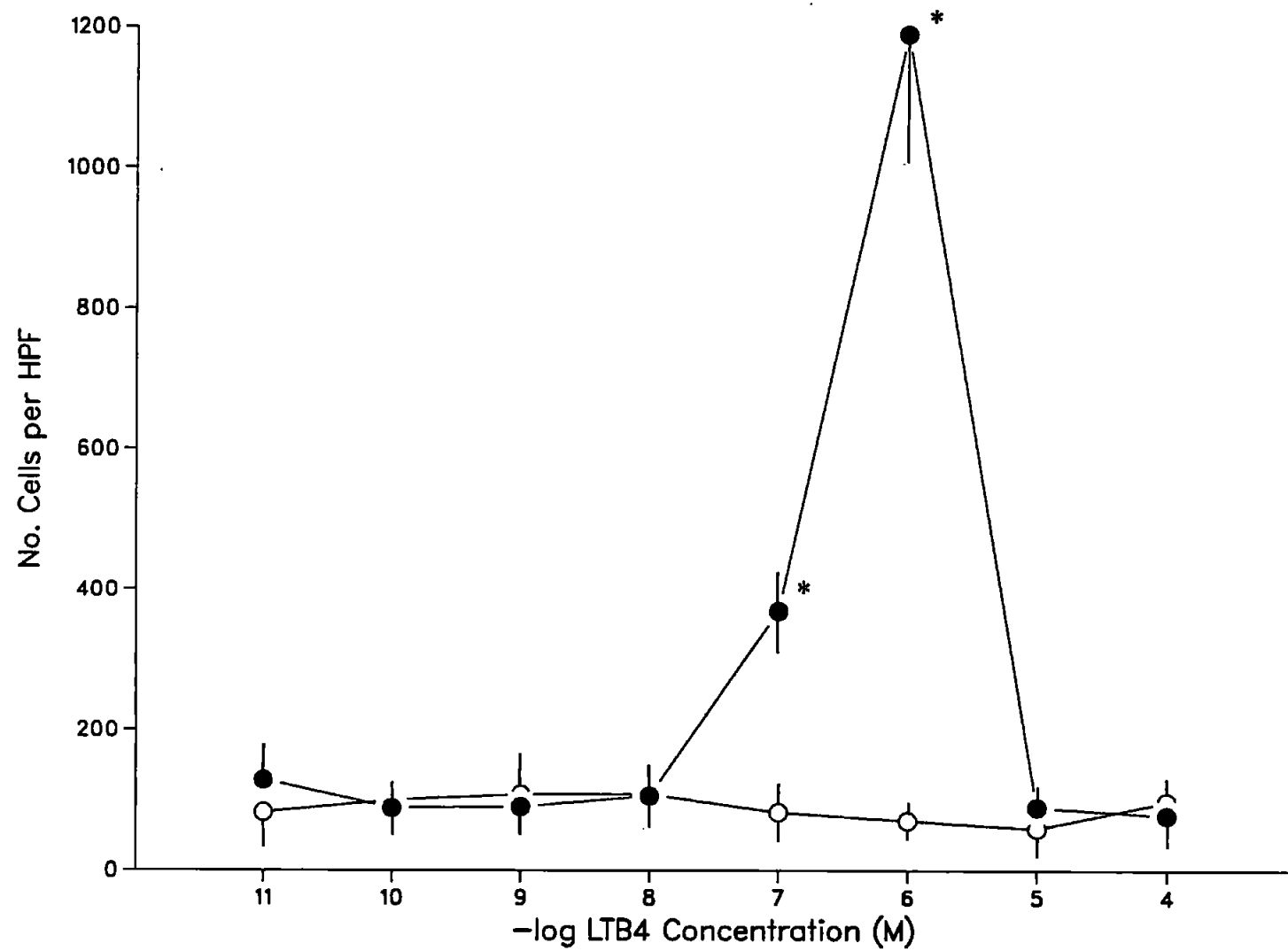
**Figure 27    Effects of Leukotriene B<sub>4</sub> on leucocyte migration using the microchemotaxis chamber.**

**The effect of various concentrations of Leukotriene B<sub>4</sub> (●) and a saline control (O) on turbot kidney leucocyte migration.**

**Symbols represent average number of migrated cells observed on underside of filter per 10HPFs in triplicate assays in each experiment**

**( | indicates  $\pm$ SE obtained from 3 fish)**

**Significantly different points are marked \* ( $p \leq 0.01$  compared with control).**



### 7.3.3 CAPILLARY TUBE MIGRATION ASSAY

Distances travelled by leucocytes migrating from a capillary tube in the presence of various concentrations of a 24h culture filtrate of *V. alginolyticus* (Figure 28) were measured and compared. These results are given in Table 8. No significant enhancement of migration was seen at any supernatant concentration tested ( $p \leq 0.01$ ). Significant inhibition of migration was evident however, at concentrations of 10% and 50%.

### 7.3.4 LEUCOCYTE POLARISATION ASSAY

Polarisation results were determined by the visual scoring of the morphological appearance of leucocytes from each experimental condition and expressed as a % of the 300 cells scored for each data point falling in the polarised or non-polarised categories. Attractants tested included *V. alginolyticus* culture supernatant, normal turbot serum and casein. Results are given in Table 9.

Polarisation was significantly higher than controls in cell suspensions stimulated using *V. alginolyticus* supernatant, *V. alginolyticus* control medium and normal turbot serum. Maximum polarisation occurred in response to *V. alginolyticus* supernatant. Polarisation in response to *V. alginolyticus* supernatant was also significantly higher than to that of *V. alginolyticus* control medium ( $p \leq 0.01$ ).

No significant polarisation was seen in response to casein.

Polarised cells were identified as granulocytes following Sudan black B staining (Figure 29).

Figure 28    Leucocyte migration in the capillary tube migration assay.

Migration of turbot kidney leucocytes in response to a 50% 24h *Vibrio alginolyticus* culture supernatant.

- CT    Capillary Tube
- L    Migrating Leucocytes

(Bar represents 500µm)

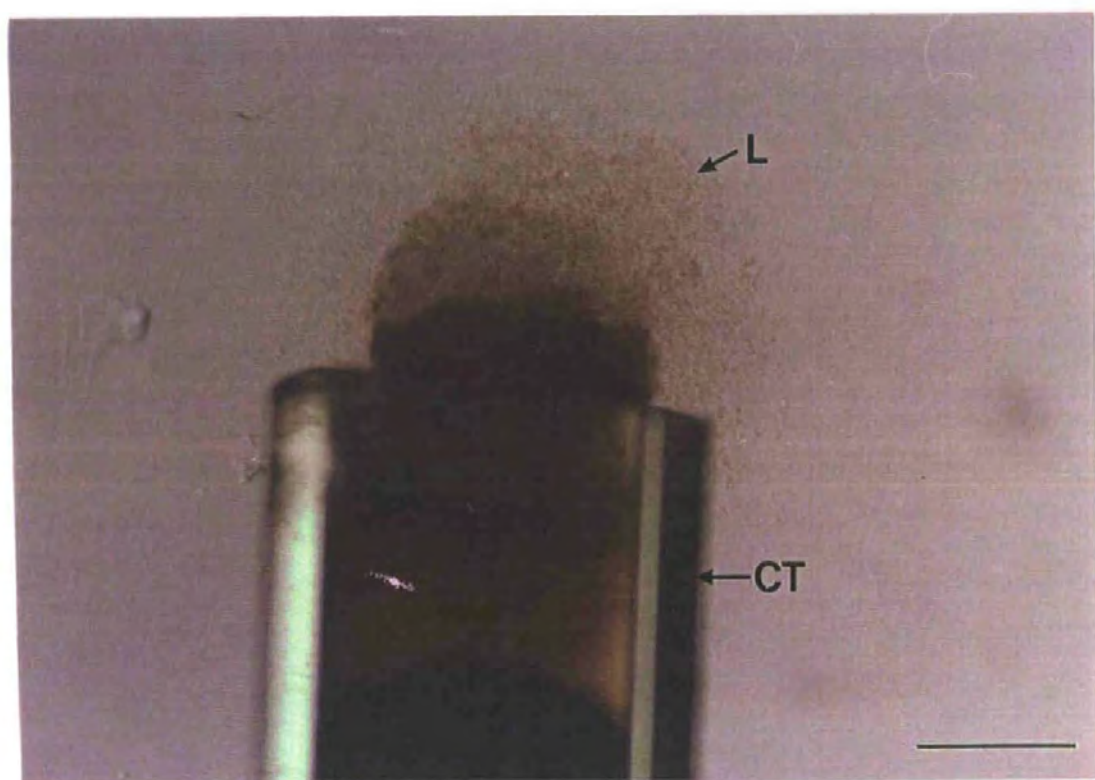




Table 8

MIGRATION OF TURBOT KIDNEY LEUCOCYTES IN RESPONSE TO A 24H *VIBRIO ALGINOLYTICUS* CULTURE SUPERNATANT USING THE CAPILLARY TUBE MIGRATION ASSAY.

ATTRACTANT	MIGRATION DISTANCE
Bacterial supernatant	(mm)
50%	0.65 (± 0.4) *
10%	1.32 (± 0.7) *
1%	1.89 (± 1.1)
0.1%	2.16 (± 0.5)
Control	1.94 (± 0.4)

Figures represent mean average measurements (± SD) of 3 observations from each of 5 fish.

\* indicates significant inhibition (p ≤ 0.01)

Table 9

THE EFFECTS OF A VARIETY OF CHEMOATTRACTANTS ON  
TURBOT KIDNEY LEUCOCYTES USING THE POLARISATION  
ASSAY.

STIMULUS (a)	% POLARISED GRANULOCYTES (b)	
<i>Vibrio alginolyticus</i> supernatant (100%)	66.1 ( $\pm$ 8.4)	*
Normal Turbot Serum	49.2 ( $\pm$ 4.1)	*
Casein (5mgml <sup>-1</sup> )	22.2 ( $\pm$ 2.1)	
<i>Vibrio alginolyticus</i> control	33.9 ( $\pm$ 5.4)	*
Control (1.1% Saline)	17.3 ( $\pm$ 4.8)	

(a) Cells incubated for 30min at 20°C with indicated concentration of chemoattractant stimuli and the level of polarisation determined.

(b) Figures represent mean average Polarisation values ( $\pm$  SD) of 3 observations of 300 leucocytes from each of 3-5 fish.

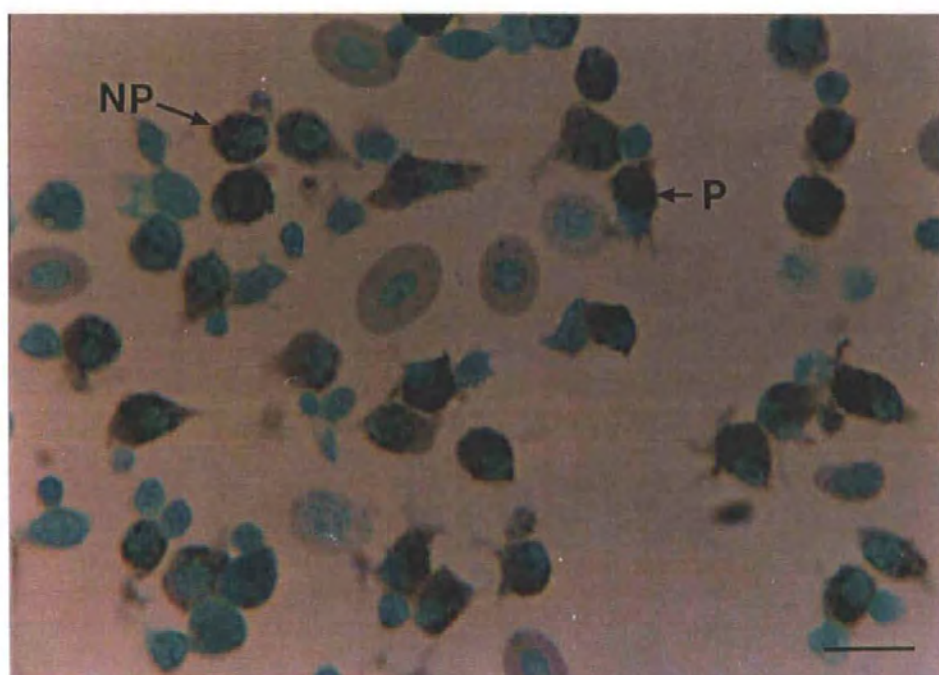
\* indicates significant difference ( $p \leq 0.01$ )

Figure 29    Leucocyte polarisation *in vitro*.

Polarised turbot granulocytes in response to 100% *Vibrio alginolyticus* culture supernatant in the leucocyte polarisation assay. (Sudan black B / Methyl Green).

- P     Polarised granulocyte
- NP   Non-polarised granulocyte

(Bar represents 10µm)



## CHAPTER 7

### 7.4 DISCUSSION

This study has demonstrated that turbot leucocytes exhibit *in vitro* migration in response to the chemostimulatory substances; Leukotriene B<sub>4</sub>, serum and bacterial culture filtrates suggesting these may be important mediators in the cellular migratory responses of the turbot.

Migration of turbot leucocytes was shown here to be influenced by the growth products of *V. alginolyticus* at dilutions between 5% and 50%. However, a marked reduction of leucocyte migration and in some cases inhibition, occurred at the highest concentrations of bacterial growth products. Similar results were obtained for migration of plaice kidney leucocytes to the culture products of this bacteria (Nash *et al*, 1986). This phenomenon of cellular attraction at low concentrations and inhibition at higher concentrations has been reported in mammals (Jungi, 1977). He concluded that a chemotactic signal can elicit either unidirectional migration or inhibition of migration at different concentrations. Such responses may provide a mechanism whereby cells are attracted towards a site of bacterial invasion and once at the infected site, migration is inhibited resulting in cellular accumulation within the foci of infection. However, the possibility of cell damage caused by the release of bacterial extracellular products cannot be ruled out as an alternative explanation for the reduced cell numbers seen at high concentrations of bacterial supernatant.

Turbot leucocytes were unresponsive to FMLP, irrespective of the concentration. In view of the response seen to *V. alginolyticus*, this is perhaps surprising as FMLP is a synthetic analogue of bacterial peptides known to be present in bacterial secretions (Marasco *et al*, 1984). Nonetheless, these findings are supported by those of Hunt and Rowley

(1986b) who were unable to detect any chemotactic/kinetic response in dogfish (*Scyliorhinus canicula*) leucocytes to FMLP using the under agarose assay. In contrast, Nash *et al* (1986) found that purified plaice neutrophils were responsive to FMLP using the microchemotaxis chamber assay. These findings suggest that turbot and dogfish leucocytes may lack FMLP receptors suggested to be present in the plaice or may reflect inherent differences between the techniques employed.

Both normal and zymosan activated serum were shown to stimulate migration of turbot leucocytes when the microchemotaxis chamber method was employed. Such a response may be indicative of complement involvement. Chemotactic activity in mammals has been associated with the C5a component of complement (Damerau *et al*, 1980) and in view of the findings of Nonaka *et al* (1981b) that rainbow trout possess a C5-like molecule, it is possible that such a component is involved in teleostean leucocyte migration. Additional evidence for the involvement of C5 was provided by Obenauf and Hyder Smith (1985) who proposed that the migration of nurse shark leucocytes to normal rat serum was due to the presence of C5a receptors on the leucocyte surfaces.

Migration in the present study was reduced in response to heat inactivated serum, further suggesting a role for complement in this response. Boyden (1962) found that heating serum inhibited chemotactic activity and suggested the involvement of complement. However, the fact that there was a migratory response with heat inactivated serum suggests that factors other than complement alone may play a role in mediating cell migration in the turbot. The observation here of enhanced migration in response to normal serum above that seen for activated serum at all but the lowest concentrations may be due to the ease with which serum can be activated, with the process of producing and handling it being sufficient to cause some activation.

The under agarose assay failed to detect any significant migration of turbot leucocytes to these sera. In support of this finding, Gee (1984) was unable to detect definite migration to activated serum using the under agarose assay in mammals, attributing this to the relative insensitivity of the method. This suggests that the microchemotaxis chamber system provided greater sensitivity than the under agarose assay when used in the present study. Leucocyte polarisation was, however, observed in the presence of normal turbot serum which contrasts the findings of Hunt and Rowley (1986b) who found dogfish granulocytes unresponsive to serum factors. This may indicate differences in the mechanisms of polarisation/migration between these species.

Elevated levels of cellular migration were induced by  $LTB_4$  in the turbot. This substance is known to be one of the most active of the lipoxygenase activated derivatives of arachadonic acid and an extremely potent chemoattractant, producing a rapid accumulation of leucocytes when given locally *in vivo* (Turner and Lynn, 1978; Goetzl and Pickett, 1980). That similar enhancement was also obtained with dogfish (Hunt and Rowley, 1986b) and plaice leucocytes (Nash, Fletcher and Thomson, 1986) in response to  $LTB_4$ , may suggest a role for this group of chemicals in the inflammatory responses of fish, as in mammals.

Turbot leucocytes failed to respond to casein in any of the assays used in the present study and it is possible that this reflects an absence of the relevant cell surface receptors in this species, since casein is a very potent mammalian chemoattractant (Keller and Sorkin, 1976).

As it was not possible to prepare pure cell populations using a Percoll density gradient, cell suspensions used in the *in vitro* assays in this study contained several leucocyte types. From the examination of stained microchemotaxis chamber filters, migratory cell populations appeared to be predominantly

composed of granulocytes. Sudan black B staining of polarised cells further suggested these cells to be granulocytes. However, a role in the turbot of other cell types cannot be ruled out. Chemotactic cells were identified as neutrophils and macrophages in the plaice (MacArthur *et al*, 1985), neutrophils in the eel (Suzuki, 1986) and granulocytes in the dogfish (Hunt and Rowley, 1986b), all of which are cell types which have been associated with inflammatory responses in fish (Finn and Neilson, 1971; Timur *et al*, 1977; Timur and Roberts, 1977).

Of the methods employed here to assess leucocyte stimulation *in vitro*, the microchemotaxis chamber system proved most sensitive and easily quantifiable. It also had great advantages over standard migration chambers in that reduced diffusion distances resulted in much shorter incubation times and the requirement for smaller volumes, allowing many replicates to be performed simultaneously. Furthermore, specialised counting methods (Checkerboard Assay) can be employed to distinguish between chemotactic and chemokinetic migration (Zigmond and Hirsch, 1973). One drawback of this method is that no information can be gained on morphological changes occurring during migration.

The under agarose assay enabled simultaneous examination of cell responses to both the attractant under test and the control within the same cell population. However, it was influenced by a large number of variables, including gel type and concentration, distance between wells and migration substrate. Although most of these variables can be standardised, differences in diffusion rates between different chemoattractants is difficult to control and will hinder their direct comparison. In this study, a 0.8%, type IV agarose gel proved to be the optimum compromise between maximising cell migration and gel manipulation. Although relatively rapid, easily quantifiable and inexpensive, the usefulness of the under agarose technique was found to be limited, since responses were only consistently detected in response to



very high concentrations of potent attractants. Indeed, Gee (1984) stated that this method was 100 times less sensitive than filter assays, recommending that direct comparisons of responses obtained with different *in vitro* assays should be treated cautiously.

In the search for reliable and easily quantifiable assays, two further assays, namely the capillary tube migration and leucocyte polarisation assays were assessed using bacterial culture filtrates. The capillary tube migration assay was quicker to evaluate than the filter based assay but problems were encountered obtaining a "clean break" of the capillary tube at the leucocyte interface and there was a tendency for patterns of migrated leucocytes to be disrupted during the removal of coverslips and liquid surrounding migrated cells. The polarisation assay was inexpensive, easy to perform and rapid. The "immediate" nature of the polarising response makes this a useful assay which would greatly simplify the screening of chemoattractants if the reproducibility found in mammalian studies can be transferred to the *in vitro* assessment of fish leucocyte migration.

Ultimately, the choice of assay system depends upon the questions being asked and the practical requirements of the investigator. Since neither filter-based, nor direct observation assays alone provide complete information on cell locomotion, the use of both types of test in parallel is recommended. The ability of the assays tested here to detect migration varied considerably even in response to the same chemoattractant, highlighting the need to exercise caution when comparing results obtained by different methods. Care must be exercised as laboratory assays of chemotaxis often provide over simplified models for the locomotion of cells into inflammatory sites with tests being chosen for their convenience rather than sensitivity and closeness to *in vivo* situations. From the results shown here, the microchemotaxis chamber assay in conjunction with the polarisation assay would appear to be rapid and sensitive techniques which can be used to

provide an insight into the inflammatory process and the assessment of immunocompetence of stressed and diseased fish.

## CHAPTER 8

### GENERAL OVERVIEW

Significant steps towards increasing our fundamental knowledge and understanding of the cellular elements involved in the defence mechanisms of the turbot have been made in this study. Turbot seem to possess many of the elements of the cellular immune system described for other fish species. The leucocytes have been identified and aspects of their migratory and phagocytic capabilities ascertained. The structure and development of the lymphoid tissues have also been described along with their role in the clearance of foreign particulate matter.

The major lymphoid organs of the turbot, as in other fish, were the thymus, kidney and spleen. The ontogeny of these organs was found to follow a similar pattern to that reported in other fish with the exception that the spleen appeared to be present at a much earlier stage of development. Both the spleen and the kidney were instrumental in the clearance of particulate matter from the bloodstream via the fixed cells of the RES, which were predominantly macrophage-like in appearance. Since little intracellular carbon was seen in blood-borne phagocytes, their role in this clearance appeared to be minimal.

Four distinct leucocyte types were identified, namely thrombocytes, lymphocytes, granulocytes and monocytes. Generally speaking, these were morphologically, ultrastructurally, histochemically and functionally similar to their counterparts in other fish species and in mammals.

Thrombocytes and monocytes were found to contain intracellular organelles and histochemical properties indicative of a capacity for migration and phagocytosis. Thrombocytes in particular were highly phagocytic *in vitro* and *in vivo*. Furthermore, thrombocytes represent a much higher proportion

of the leucocyte population of the turbot than do granulocytes, which is in contrast to the high proportion of granulocytes seen in higher vertebrates. In respect of this, it is proposed that the turbot thrombocyte may undertake some of the functions of mammalian granulocytes, having a dual role in clotting and clearance of particulate matter.

Only one granulocyte type was identified in the turbot with functional and morphological characteristics of the mammalian neutrophil but lacking a demonstrable phagocytic capacity *in vitro*. However, it is felt that this absence of activity reflects sub-optimal experimental conditions for this cell type *in vitro*, since uptake of carbon by these cells, although limited, was observed *in vivo*. Granulocytes, the primary migratory cell type, exhibited responses to *Vibrio alginolyticus* culture supernatant, Leukotriene B<sub>4</sub> and turbot sera. Involvement of complement in the induction of enhanced leucocyte migration was suggested by the results of this study, although a role for a heat stable serum factor and pathogen derived factors was also demonstrated.

The combination of such phagocytic and migratory properties suggests that these cells are important components of the inflammatory response of the turbot, migrating to sites of injury and/or invasion, phagocytosing cell debris and foreign particulate material.

Building upon the results presented here, further work to study the role of lymphocytes in cell-mediated reactions of the turbot would usefully include the investigation of soluble factors produced in culture supernatants following antigenic and mitogenic stimulation. Characterisation of the effects of such factors on cellular responses could then be undertaken. Only then might it prove possible to assess the influence of various vaccination strategies upon the cellular immune responses of fish.

## APPENDIX I

### HIGH PROTEIN FISH DIET

#### Complete mineral mix:

(Lindsay *et al*, 1984)

	(gkg <sup>-1</sup> ):
Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub> .H <sub>2</sub> O	590
NaH <sub>2</sub> PO <sub>4</sub> .2H <sub>2</sub> O	210
MgCO <sub>3</sub>	30
FeSO <sub>4</sub> .7H <sub>2</sub> O	30
KCl	50
NaCl	80
Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> .16H <sub>2</sub> O	0.2
ZnSO <sub>4</sub> .7H <sub>2</sub> O	4.0
CuSO <sub>4</sub> .5H <sub>2</sub> O	1.0
MnSO <sub>4</sub> .4H <sub>2</sub> O	3.6
KI	0.2
CoSO <sub>4</sub> .7H <sub>2</sub> O	1.0

#### Vitamin mix:

(Cowey *et al*, 1981)

Thiamine HCl	2.665g
Riboflavin	10.660g
Pyridoxine HCl	2.665g
Choline Bitartrate	483.550g
Nicotinic Acid	39.975g
Ca Pantothenate	26.650g
Inositol	106.600g
Biotin	266mg
Folic Acid	799mg
Ascorbic acid	53.300g
Vitamin B12(in $\alpha$ cellulose)	10.000g ( $\equiv$ 5mg Vit B12)
Menadione	2.132g
$\alpha$ Topophenol Acetate	2.100g
(or Vit E 50)	(or 4.200g)

(Make up to 1,500g with  $\alpha$  cellulose)

This was roughly mixed in a large plastic bag.

### **Moist pellets contained;**

White fish meal (Norsabel)	4410g
Fish oil (Solvitex)	100g
Carboxymethylcellulose (Blannose)	250g
Complete mineral mix	100g
Vitamin mix	140g
Water	1900g

1. Mix dry ingredients together thoroughly for 30min.
2. Add oil slowly and evenly.
3. Add water and mix for just enough time to allow the ingredients to combine (2min).
4. Extrude mixture through a mincer with a die which will produce pellets of 3mm diameter.
5. Freeze food at -20°C until required.

## **APPENDIX II**

### **JUVENILE FOOD SUPPLEMENT**

Fresh fish	200g (wet weight) or 100g (dry weight)
Alginate	1.0g
Vitamin mix	1.0g
Cod liver oil	8%

1. Flake fish to produce small fragments.
2. Mix other ingredients together thoroughly.
3. Freeze at -20°C until required.

## **APPENDIX III**

### **MAY-GRUNWALD AND GIEMSA STAIN (MGG)**

(For thin blood films or tissue imprints)

1. Fix slide for 5 - 10min in methanol.
2. Pipette neat May Grunwald stain (BDH) onto the microscope slide and leave for 5min.
3. Add an equal volume of distilled water to the slide and mix water and stain by rocking the slide about its axis. Leave for a further 5min.
4. Pour off stain and add sufficient of a 1:10 dilution of Giemsa stain (BDH) to cover the smear. Leave for 10min.
5. Pour off stain, rinse slides rapidly in distilled water and dry immediately in an airstream.
6. Check staining and mount using DPX mountant (R A Lamb).

#### **Results:**

Cytoplasm colour - red / purple  
Nuclei colour - blue



## **APPENDIX IV**

### **FORMAL BUFFERED SALINE**

Formalin	500ml
Sodium dihydrogen orthophosphate	22.75g
Disodium hydrogen orthophosphate	32.50g
Sodium chloride	22.50g
De-ionised water	2l

(All chemicals were obtained from BDH Chemicals Ltd, Poole, UK).

Fix tissues in approximately 10 x volume fixative for 7 days due to the relatively slow penetration of this fixative.

## **APPENDIX V**

### **TREATMENT OF TISSUES IN AN AUTOMATIC TISSUE PROCESSOR**

Tissues are processed as follows:

	<b>Time</b>	<b>Vacuum</b>
70% Alcohol	18h	-
90% alcohol	9h	-
Absolute alcohol	4h	-
Absolute alcohol	3h	-
Absolute alcohol	3h	-
Xylene	1h	-
Xylene	1h	-
Xylene	1h	-
Wax	1h	+
Wax	1h	+

Place tissues in an evacuated chamber for 1h to ensure complete penetration of wax into the larger samples.

## **APPENDIX VI**

### **HAEMATOXYLIN AND EOSIN STAIN (H&E)**

(For routine haematology)

Coat slides thinly with egg albumin to ensure adhesion of sections during the bleaching process.

#### **Bleaching of sections to remove melanin:**

1. Immerse slides in 0.1% potassium permanganate solution for 12h.
2. Place directly into 0.1% Oxalic Acid for 1min without rinsing.
3. Rinse slides thoroughly in distilled water.

#### **H&E Staining**

1. Stain using Haematoxylin (Harris's, BDH, Poole) 30min, check intensity.
2. Acid alcohol for a few seconds - until the colour just starts to leach out.
3. Lithium carbonate (saturated aqueous solution) for up to 1min - until sections stain denim blue.
4. 1% Eosin in 1% Calcium nitrate (5-10sec). Check colour.
5. Rinse in distilled water.
6. 90% alcohol then bring sections to xylene and mount using DPX.

Unbleached sections of each tissue type are used to provide controls.

#### **Results:**

Cytoplasm colour - pink / red  
Nuclei colour - blue

## **APPENDIX VII**

### **SUDAN BLACK B STAIN (Sheehan and Storey, 1947)**

(For lipids)

#### **Reagents:**

- (a) Sudan black B (Gurr) 0.3g in 100ml absolute ethanol.
- (b) Dissolve 16g crystalline phenol (or 20g phenol (80% w/w) in 30ml absolute ethanol. Add to 100ml aqueous solution containing 0.3g hydrated disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ).
- (c) Working stain: Add 40ml buffer (b) to 60ml sudan black B solution (a) and filter. Keeps for 2-3 months.

#### **Technique:**

- 1. Fix air dried smears in formalin vapour or in a 1:9 formalin:alcohol mixture for 5 - 10min.
- 2. Wash in running tap water for 10min.
- 3. Immerse in working stain for 1h.
- 4. Wash off in 70% ethanol.
- 5. Wash with tap water for 2min.
- 6. Blot dry and counter stain with MGG.

#### **Results:**

Reaction products indicative of lipid can be seen within the cytoplasm and nucleus of cells as areas of intense blue / black stain.

Several other methods of this stain which did not utilise a phenol buffer were tested, without success.

## **APPENDIX VIII**

### **PERIODIC ACID SCHIFF STAIN (PAS)**

(General polysaccharide stain)

#### **Reagents:**

Periodic acid solution:

Dissolve 5g periodic acid crystals in 500ml distilled water.  
(Alternatively 1ml 50% periodic acid solution (BDH) may be used  
(1ml contains 1.6g periodic acid) in 160ml distilled water).

Store in dark bottle. Keeps for 3 months (room temperature).

(Schiff's basic fuchsin reagent (BDH) should be stored in the dark at 4°C).

#### **Technique:**

1. Fix air-dried smears for 10min in a solution of 10ml formalin (40%) and 90ml ethanol.
2. Wash briefly in tap water.
3. Treat with the periodic acid solution for 10min.
4. Wash and blot dry.
5. Immerse in Schiff's basic fuchsin in Coplin jar for 30min.
6. Wash in tap water 5 - 10min.
7. Counter stain in aqueous haematoxylin (Harris's) for 10-15min.

(Control smears are exposed to salivary amylase digestion for 30min between stages 2 and 3).

#### **Results:**

PAS positive material is indicated by the red / purple stain.

(Usually glycogen, which can be confirmed by the incubation of slide with salivary amylase prior to incubation).

## APPENDIX IX

### ACID PHOSPHATASE

#### Reagents:

Naphthyl AS-BI phosphoric acid	10mg
Fast garnet GBC salt (Gurr)	10mg
Walpole's acetate buffer, 0.1M, pH 5	50ml

Freshly prepared substrate is filtered into a Coplin jar.

#### Walpole's acetate buffer:

##### Solution A

0.2M Acetic Acid	12gl <sup>-1</sup> (Sigma)
------------------	----------------------------

##### Solution B

0.2M Sodium acetate	16.4gl <sup>-1</sup> (C <sub>2</sub> H <sub>2</sub> O <sub>2</sub> Na, BDH)
or	27.2gl <sup>-1</sup> (C <sub>2</sub> H <sub>2</sub> O <sub>2</sub> Na.3H <sub>2</sub> O, BDH)

<b>0.1M Buffer:</b>	Solution A	14.8ml
	Solution B	35.2ml

Make up to 100ml with water

#### Technique:

- 1 Fix air-dried smears in formalin vapour for 4min.
- 2 Wash briefly in tap water and blot dry.
- 3 Incubate in substrate solution at 22°C for 1 - 1.5h.
- 4 Wash briefly in tap water.
- 5 Counter stain with aqueous haematoxylin for 10 - 15min.

#### Results:

Sites of acid phosphatase activity are visualised as fine / coarse red / brown deposits.

(One of the few acid hydrolases that can be demonstrated in lymphoid cells, showing greater activity in B rather than T cells)

For good results Fast Garnet must be stored frozen at - 20°C

## APPENDIX X

### ALKALINE PHOSPHATASE

#### Reagents:

##### Stock propanediol buffer solution (0.2M):

Dissolve 10.5g of 2-amino-2-methyl propane-(1:3)-diol (Sigma) in 500ml distilled water. Store at 4°C and discard after 3 months.

##### Working buffer:

Mix 25ml of stock buffer with 5ml of 0.1N HCl and dilute with distilled water to 100ml.

##### Methyl green stain:

Methyl green	2gm
Distilled water	100ml

1. Dissolve powder in distilled water in separating flask.
2. Add 50ml chloroform and mix.
3. Leave for 48h, mixing gently 4 or 5 times (chloroform frees stain from contamination with methyl violet).
4. Store at 20°C in continuous contact with the chloroform.

##### Technique:

1. Fix air-dried smears in 10% formalin in absolute methanol for **EXACTLY** 30sec (use a stop watch) at  $0\pm5^{\circ}\text{C}$
2. Prepare substrate as follows:

Sodium alpha-naphthylphosphate (Gurr, Sigma)	35mg
Fast garnet GBC salt (Gurr, R A Lamb)	35mg
Working 0.05M propanediol buffer	35ml
3. **AS SOON AS** the substrate has been mixed, pour directly onto the slides and allow to incubate at room temperature for 5 - 10min. (The substrate must be used within 5min of preparation).
4. Rinse slides in tap water for 10sec.
5. Counter stain with methyl green for 10-15min.

##### Results:

Sites of alkaline phosphatase activity as black or brown deposits.

## APPENDIX XI

### NON SPECIFIC ESTERASE

#### Fixation

##### Buffered formalin acetone:

Sodium phosphate ( $\text{Na}_2\text{HPO}_4$ )	20mg
Potassium phosphate ( $\text{KH}_2\text{PO}_4$ )	100mg
Water - tap	30ml
Acetone	45ml
Formalin	25ml

Adjust to pH 6.6 and refrigerate

(For the preparation of the buffered formalin and Solution B, wear gloves and visor and prepare in fume cupboard)

#### Technique:

1. Fix air dried smears in buffered formalin acetone for 30sec at 4- 10°C.
2. Wash in 3 changes of distilled water, then air dry at room temperature for 10-30min.

USE ACID CLEAN GLASSWARE OR NEW DISPOSABLE BEAKERS

#### Stains:

##### Solution A:

Sorenson's Buffer (pH 7.6)	26.7ml
(check with pH meter)	

##### Solution B:

Pararosanilin hydrochloride	1gm
Distilled water	20ml
Concentrated hydrochloric acid	5ml

1. Dissolve by gentle warming.
  2. Filter after cooling.
- (Stable for several days)

##### Solution C:

Sodium nitrite	0.4g
Distilled water	10ml

(MUST be freshly made)

##### Solution D:

Alpha naphthyl acetate (Sigma)	30mg
2- ethoxyethanol	1.5ml



**Solution E:**

Sorenson's buffer (pH 7.4) 28.5ml  
(Use remaining original buffer and change pH)

**1% Methyl Green**

(see Appendix X)

**Staining procedure:**

1. Mix 0.9ml Solution B with 0.9ml Solution C and allow to stand for **1min.**
2. Add Solution A and mix well.
3. To Solution D, add solutions from step 2. Adjust pH to 6.1 and filter. **IMMEDIATELY** place slides in this solution for 45min.
4. Wash in running water for 5min.
5. Rinse in 3 changes of distilled water.

**Results:**

Non-specific "butyrate" esterase - stains dark red / brown

Non-specific esterase is a reliable marker for mammalian monocytes and macrophages (granulocytes are negative).

Distinct dot - like reaction in lymphocytes.

## APPENDIX XII

### PREPARATION OF TISSUES FOR TRANSMISSION ELECTRON MICROSCOPY (TEM)

All reagents and resins used in EM are particularly hazardous and should be used with **extreme caution**.

#### Cacodylate Buffer (0.2M):

Sodium Cacodylate (Fisons) 42.806g

Make up to 1l with distilled water

#### Fixative:

Buffer + 3% gluteraldehyde

#### Resin

1mm<sup>3</sup> tissue samples were fixed and embedded as follows:

1.	Fixative		2.5h	4°C
2.	Wash in buffer	1	0.5h	4°C
		2	0.5h	4°C
		3	0.5h	4°C
3.	Osmicate (OsO <sub>4</sub> )		1.5h	4°C
4.	Wash in buffer	1	0.5h	4°C
		2	0.5h	4°C
		3	0.5h	4°C
			(overnight if necessary)	
5.	50% ethanol		0.5-2h	4°C
6.	70% ethanol		0.5-2h	4°C
			(overnight if necessary)	
7.	90% ethanol		0.5-2h	4°C
8.	Ethanol	1	0.5-2h	4°C
		2	0.5-2h	4°C
		3	0.5-2h	

Bring to room temperature (RT) on turner

- |     |                |       |    |
|-----|----------------|-------|----|
| 9.  | Ethanol: resin |       |    |
|     | 3 : 1          | 1-12h | RT |
|     | 2 : 2          | 1-12h | RT |
|     | 1 : 3          | 1-12h | RT |
| 10. | Pure resin     | 1-12h | RT |
11. Embed and label samples in beam capsules or coffins using fresh resin.
12. Polymerise resin in oven at 40°C for 24h.

## APPENDIX XIII

### PLAICE RINGER

(Cobb *et al*, 1973)

NaCl	9.00g
KCl	0.39g
NaHCO <sub>3</sub>	23.0g
NaH <sub>2</sub> PO <sub>4</sub> .2H <sub>2</sub> O	0.032g
Na <sub>2</sub> HPO <sub>4</sub> .2H <sub>2</sub> O	0.248g
MgCl.6H <sub>2</sub> O	0.12g
CaCl <sub>2</sub> .6H <sub>2</sub> O	0.55g
Glucose	1.00g

Double distilled water    1000ml

## **APPENDIX XIV**

### **GELATINE COATED SLIDES**

#### **Gelatine Coating:**

1. Dissolve 5g gelatine (BDH) in 1l boiling distilled water with stirring.
2. Add 0.5g Chrome alum (BDH).
3. Allow to cool and store at 4°C for up to 1 week until required.

#### **To coat the slides:**

1. Soak slides in concentrated hydrochloric acid for 24h.
2. Rinse in running tap water for 24h.
3. Rinse in several changes of distilled water.
4. Dry in slide racks in incubator.
5. Dip each slide in the gelatine solution and allow to drain at an angle of 45° to the bench for several hours or overnight.
6. Store at 4°C until required.

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